

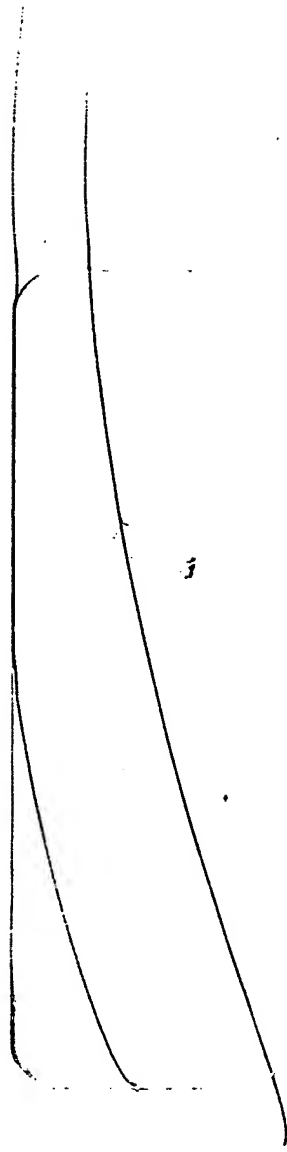
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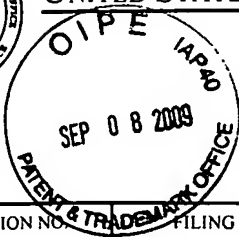
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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10/579,088

01/14/2008

Manzer Durrani

6451

7590 08/27/2009
MANZER DURRANI
8290 CLEARY BLVD., VILLA #2906
PLANTATION, FL 33324

EXAMINER

KAUFMAN, CLAIRE M

ART UNIT	PAPER NUMBER
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1646

MAIL DATE	DELIVERY MODE
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08/27/2009

PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/579,088	DURRANI ET AL.	
	Examiner	Art Unit	
	CLAIRE KAUFMAN	1646	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 13 May 2009.
- 2a) ☐ This action is FINAL. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-32 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-32 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☒ Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date 1/14/08, 4/21/08, 8/24/09.
- 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____.
- 5) ☐ Notice of Informal Patent Application
- 6) ☐ Other: _____.

DETAILED ACTION

Claim Rejections - 35 USC § 112, Second Paragraph

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1, 5, 7, 19, 28-32 and dependent claims are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 1, 5, 7, 19, 28 and 30 are unclear because of the use of the terms “native” and “variant”. While the specification says that ““native AAT” (alpha 1-antitrypsin) refers to AAT forms that can be isolated from natural sources” (p. 4, lines 19-20 [0020], variant AAT refer to functional equivalents to the native (p. 4, line 34) and “proteins that are substantially identical to a native sequence.” (p. 5, line 6) Also, native AAT includes allelic and splice variants as well as truncated forms (p. 4, lines 21-22). Because of the overlap in definitions, the metes and bounds of variant vs. native AAT cannot be determined.

Claims 28-30 are duplicates of claims 5-7 and claims 31-32 are duplicates of claims 8-9.

Claim Rejections - 35 USC § 112, First Paragraph

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-32 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for an AAT which is a serine protease inhibitor, does not reasonably provide enablement for an AAT which does not have serine protease inhibitory activity. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

Art Unit: 1646

The claims are drawn to a pharmaceutical composition comprising an AAT, which is a native, recombinant or variant AAT, in addition to other non-protein components. Because the composition is a "pharmaceutical composition", it must have therapeutic use. Wildtype AAT is recognized as a serine proteinase (or protease) inhibitor (US 5,166,134, col. 2, lines 4-25, IDS of 1/14/08). There is no structural or functional limitation of the AAT in the claims. That is, the AAT is not claimed by specific sequence, for example, which would inherently confer a particular function or have an explicit functional requirement. It is acknowledged that there are over 100 AAT naturally occurring genetic variants known (Luisetti et al., Thorax, 59:164-9, 2004). However, the claims including an "AAT variants" include not only functional variants, but sequence variants with substitutions, deletions and/or insertions relative to a native sequence (which includes allelic and splice variants). Single amino acid changes effect the function of AAT. Van Steenberg (Acta Clin. Belgica, 43:171, 1993) reports (paragraph beginning p. 176, col. 2) that substitution of Glu342 -> Lys342 results in a deficiency variant in which "85% of the normally synthesized polypeptide is blocked in the endoplasmic reticulum..." "Glu264 -> Val264 ...does not lead to intracellular accumulation but to an early intracellular proteolysis..." of the nascent S polyp This is pharmacologically important because mutation of these residue can lead to significantly decreased plasma levels and increased risk of emphysema and liver disease (*ibid.*). Carrell et al. (Nature, 1982, IDS filed 1/14/08) showed that two AAT variants are linked to progressive loss of lung elasticity that contributes to lung damage such as emphysema (*e.g.*, p. 33, col. 2, second paragraph).

Native variants have been characterized as "normal, deficient, null and dysfunctional" (Ljubic et al., J. Biochem. Biophys. Meth. 68(3):167-173, 2006, p. 168, end of second full paragraph). Because one skilled in the art would not reasonably expect that an AAT which was not a proteinase inhibitor could be of therapeutic benefit, and because the claims encompass an AAT with no or reduced serine protease inhibitor function, the invention is not enabled for the full breadth of the claims. That is, an AAT with reduced activity compared to the normal wildtype AAT would be expected to increase a subject's risk of lung and/or liver disease and the specification has not taught how to therapeutically use such AAT molecules.

Art Unit: 1646

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 19, 20, 22, 28-30 are rejected under 35 U.S.C. 102(b) as being anticipated by US 5,618,786 (IDS filed 1/14/08).

US 5,618,786 teaches an aerosol formulation in which recombinant AAT (col. 4, lines 16-17) is in an amount to provide 1 µg to 10mg/kg of host and includes the addition of lactose (a carbohydrate; col. 3, line 10 and 16-17) from 0-80% w/v, and surfactant (*e.g.*, a diglyceride) from 10-50% w/v (col. 3, lines 10-18).

Note that because there is nothing to distinguish the structure of a native, variant and recombinant AAT in the claims, the AAT taught in US 5,618,786 appears to anticipate any/all AATs.

Claims 1-7, 10-12, and 28-30 are rejected under 35 U.S.C. 102(b) as being anticipated by US 6,267,958.

US 6,267,958 teaches a composition, which may be lyophilized that comprises AAT (col. 6, lines 49), a carbohydrate called a “lyoprotectant” such as sucrose or trehalose (col. 9, lines 21-33), a surfactant such as polysorbate 80 (col. 15, lines 36-41) and an antioxidant such as methionine (col. 16, lines 5-9). The composition is also taught reconstituted with a diluent, which includes water (*e.g.*, col. 2, lines 20-23).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Art Unit: 1646

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 8-9, 13-27 and 31-32 are rejected under 35 U.S.C. 103(a) as being unpatentable over US 6,267,958 as applied to claims 1-7, 10-12, and 28-30 above, and further in view of US 5,166,134 (IDS filed 1/14/08).

US 6,267,958 teaches a composition, which may be lyophilized that comprises AAT (col. 6, lines 49), a carbohydrate called a "lyoprotectant" such as sucrose or trehalose (col. 9, lines 21-33), a surfactant such as polysorbate 80 (col. 15, lines 36-41) and an antioxidant such as methionine (col. 16, lines 5-9). The composition is also taught reconstituted with a diluent, which includes water (e.g., col. 2, lines 20-23). Also taught is a formulation wherein the protein concentration is at least 50 mg/ml (col. 2, lines 30-33). Further, an example of a reconstituted powder is shown wherein the protein is a HER2 antibody at a protein concentration for the prelyophilized formulation of 25 mg/ml, the carbohydrate (trehalose) concentration is 60 mM and the surfactant (Tween 20, a.k.a. polysorbate 20) concentration is 0.01% (Figs. 1 and 6). US 6,266,958 does not teach glycosylation state for AAT, carbohydrate concentrations as w/v or antioxidant concentrations.

US 5,166,134 teaches a pharmaceutical composition comprising AAT at 0.1-4.5% w/v in an aqueous solution (col. 2, lines 58-61). AAT is taught as glycosylated or unglycosylated (col. 3, lines 15-18). Also taught is the formulation comprising an antioxidant (col. 4, lines 22-23) and sorbitol solution (a surfactant, col. 4, line 26). AAT is taught (col. 2, lines 9-15) as having a "role in controlling tissue destruction by endogenous serine proteinases. A genetic deficiency of alpha-1-proteinase inhibitor [AAT], which accounts for 90% of the trypsin inhibitory capacity in blood plasma, has been shown to be associated with the development of asthma and pulmonary emphysema." Recombinant AAT proteins and analogs prepared by site-directed mutagenesis are taught (col. 3, lines 41-47).

Art Unit: 1646

US 5,618,786 teaches an aerosol formulation in which recombinant AAT (col. 4, lines 16-17) is in an amount to provide 1 μ g to 10mg/kg of host and includes the addition of lactose (a carbohydrate; col. 3, line 10 and 16-17) from 0-80% w/v, and surfactant (*e.g.*, a diglyceride) from 10-50% w/v (col. 3, lines 10-18). AAT is taught for the treatment of, for example, emphysema and may be isolated from a natural source, prepared recombinantly or may be a mutant of the naturally occurring form (col. 2, lines 43-50). It is stated (col. 3, lines 1-4) that, "The aerosol formulation may be varied widely, depending on the nature of the therapeutic agent, whether additional agents will be included, the manner and area in which it will be released in the lungs, or the like."

It would have been obvious to the artisan of ordinary skill at the time the invention was made to have had a pharmaceutical composition comprising AAT as taught by each of the three patents cited above and further comprising a carbohydrate (*e.g.*, trehalose), surfactant (*e.g.*, polysorbate 80) and antioxidant (*e.g.*, methionine) as taught by US 6,266,958 and US 5,618,786. Such a formulation would have been desirable because of its therapeutic application for emphysema as taught by US 5,618,786. It would have been desirable for the formulation to be in a powder (solid) or liquid form. US 6,267,958 teaches lyophilized forms which are notable for their stability and ability to retain activity in a reconstituted aqueous form. It would be obvious to have had the AAT in a glycosylated or unglycosylated form produced by isolation from nature or recombinantly as taught by US 5,166,134. It reasonably appears that the ranges of carbohydrate and surfactant concentrations taught by the prior art meet the limitations of the instant claims. Additionally, for pharmaceutical compositions, optimization of concentrations within a formulation were routine.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Claire Kaufman, whose telephone number is (571) 272-0873. Dr. Kaufman can generally be reached Monday, Tuesday, Thursday and Friday from 9:30AM to 2:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol, can be reached at (571) 272-0835.

Art Unit: 1646

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (571) 272-1600.

Official papers filed by fax should be directed to (571) 273-8300. NOTE: If applicant *does* submit a paper by fax, the original signed copy should be retained by the applicant or applicant's representative. NO DUPLICATE COPIES SHOULD BE SUBMITTED so as to avoid the processing of duplicate papers in the Office.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Claire Kaufman, Ph.D.

/Claire Kaufman/

Patent Examiner, Art Unit 1646

August 25, 2009

Receipt date: 01/14/2008

SHEET 1 OF 3

INFORMATION DISCLOSURE STATEMENT PTO-1449		ATTY. DOCKET NO. 39042-0036		SERIAL NO. 10/579,088			
		APPLICANT: Manzer Durrani					
		FILING DATE: 11/11/2004		GROUP: 1646			
U.S. PATENT DOCUMENTS							
EXAMINER'S INITIALS	PATENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE	
	4,150,071	04-17-79	Pecina				
	4,198,969	04-22-80	Virag				
	4,253,468	03-03-81	Lehmbeck				
	4,301,970	11-24-81	Craighero				
	4,453,542	06-12-84	Hughes				
	4,599,311	07-08-86	Kawasaki				
	4,620,670	11-04-86	Hughes				
	4,732,973	03-22-88	Barr, et al.				
	4,931,373	06-05-90	Kawasaki, et al.				
	5,093,316	03-03-1992	Lezdey, et al.				
	5,134,119	07-28-92	Lezdey, et al.				
	5,150,071	09-22-92	Bouzidi				
	5,166,134	11-24-92	Lezdey, et al.				
	5,218,091	06-08-93	Kawasaki, et al.				
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	4,711,848	12-08-87	Insley, et al.				
	5,780,440	07-14-98	Lezdey, et al.				
	5,993,783	11-30-99	Eljamal, et al.				
	2001/006939	07-05-01	Niven, et al.				
FOREIGN PATENT DOCUMENTS							
EXAMINER'S INITIALS	PATENT NO.	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
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EXAMINER			DATE CONSIDERED				

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

SV 2322423 v1
1/14/08 12:49 PM (39042-0036)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /CK/

INFORMATION DISCLOSURE STATEMENT PTO-1449	ATTY. DOCKET NO. 39042-0036	SERIAL NO. 10/579,088
	APPLICANT: Manzer Durrani	
	FILING DATE: 11/11/2004	GROUP: 1646
OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)		
	Carlson, et al., J. Clin. Invest., Vol. 83, pp. 1183-1190, 1989	
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	Practical Approaches to Protein Formulation Development, Byeong Chang and Susan Henderson, Rational Design of Stable Protein Formulations: Theory and Practice, Edited by John F. Carpenter and Mark C. Manning, Pharmaceutical Biotechnology Vol. 13, Kluwer Academic/Plenum Publishers, pp. 1-20, 2002	
EXAMINER		DATE CONSIDERED

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INFORMATION DISCLOSURE STATEMENT PTO-1449	ATTY. DOCKET NO. 39042-0036	SERIAL NO. 10/579,088
	APPLICANT: Manzer Durrani	
	FILING DATE: 11/11/2004	GROUP: 1646
OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)		
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	Smith, et al., J. Clin. Invest., Vol. 84, pp. 1145-1154, 1989	
	Surfactant-Protein Interactions, Theodore W. Randolph, LaToya S. Jones, Rational Design of Stable Protein Formulations: Theory and Practice, Edited by John F. Carpenter and Mark C. Manning, Pharmaceutical Biotechnology, Vol. 13, Kluwer Academic/Plenum Publishers, p. 159-171, 2002	
	Wells, et al., Gene, Vol. 34, p. 315-323, 1985	
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	Wu, et al., Proc. Natl. Acad. Sci., Vol. 91, pp. 9014-9018, 1994	
	Zoller, et al., Nucleic Acids Research, Vol. 10, No. 20, p. 6487, 1982	
EXAMINER	/Claire Kaufman/	DATE CONSIDERED 08/25/2009

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Receipt date: 04/21/2008

SHEET 1 OF 1

INFORMATION DISCLOSURE STATEMENT PTO-1449			ATTY. DOCKET NO. 39042-0036		SERIAL NO. 10/579,088		
			APPLICANT Manzer Durrani, et al.				
			FILING DATE 11/11/2004		GROUP: 1614 1646		
U.S. PATENT DOCUMENTS							
EXAMINER'S INITIALS	PATENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE	
FOREIGN PATENT DOCUMENTS							
EXAMINER'S INITIALS	PATENT NO.	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
						<input type="checkbox"/>	<input type="checkbox"/>
OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)							
/CK/	Jennings, Thomas A., "Lyophilization: Introduction and Basic Principles", Interpharm Press, pages 42-44, 261-279, (1999)						
EXAMINER			/Claire Kaufman/		DATE CONSIDERED		
					08/17/2009		

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Receipt date: 08/24/2009

PTO/SB/08 (2/92)
Sheet 1 of 1

Form PTO-1449 INFORMATION DISCLOSURE STATEMENT				Attorney's Docket No. ARR-0036		Application Serial No. 10/579,088		
(use several sheets if necessary)				Applicant(s) Manzer Durrani, et al.				
				Filing Date: January 14, 2008		Group Art Unit: 1646		
U.S. PATENT DOCUMENTS								
Examiner Initials	Ref. No.	Date	Document No.	Name	Class	Subclass	Filing Date (if appropriate)	
/CK/	1.	03/03/1992	5,093,316 A	Lezdey, et al.				
FOREIGN PATENT DOCUMENTS								
Examiner Initials	Ref. No.	Date	Document No.	Name	Class	Subclass	Translation	
							YES	NO
OTHER DOCUMENTS (including author, title, date, pertinent pages, etc.)								
Examiner Initials	Ref. No.	Title						
/CK/	2.	Hubbard, R.C., et al., "Anti-Neutrophil-Elastase Defenses of the Lower Respiratory Tract in Alpha 1-Antitrypsin Deficiency Directly Augmented with an Aerosol of Alpha 1-Antitrypsin," Annals of Internal Medicine, <u>111</u> (3): 206-212, 1989.						
/CK/	3.	He, J.Q., et al., "Pharmacogenomics of COPD," Current Pharmacogenomics, <u>1</u> (4): 229-243, 2003.						

EXAMINER: /Claire Kaufman/	DATE CONSIDERED: 08/25/2009
EXAMINER: Initial if citation considered, whether or not the citation conforms with MPEP 609. Draw a line through the citation if not in conformance and not considered. Include a copy of this form with next communication to applicant.	
*If an asterisk is placed beside the reference number, a copy is not provided because the reference was previously cited by or submitted to the PTO in a prior application that is identical in the statement and relied upon for an earlier filing date under 35 U.S.C. §120. 37 C.F.R. §1.98 (d).	

Notice of References Cited	Application/Control No. 10/579,088	Applicant(s)/Patent Under Reexamination DURRANI ET AL.	
	Examiner CLAIRE KAUFMAN	Art Unit 1646	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,267,958 B1	07-2001	Andya et al.	-----
*	B	US-5,166,134	11-1992	Lezdey et al.	-----
*	C	US-5,618,786	04-1997	Roosdorp et al.	-----
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Ljujic et al., Screening of alpha-1-antitrypsin gene by denaturing gradient gel electrophoresis (DGGE), J. Biochem. Biophys. Meth. 68(3):167-173, oct. 2006.
	V	Luisetti et al., Alpha1-antitrypsin deficiency 1: Epidemiology of alpha1-antitrypsin deficiency, Thorax, 59(2):164-169, Feb. 2004.
	W	Van Steenbergen, W. Alpha 1-antitrypsin deficiency: an overview, Acta Clin. Belg. 48(3):171-189, 1993.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.



REG-14762929

VAUZGE

NLM -- W1 AC7835 (Gen)

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Screening of alpha-1-antitrypsin gene by denaturing gradient gel electrophoresis (DGGE)

Mila Ljubic ^{*}, Aleksandra Nikolic ¹, Aleksandra Divac ¹,
Valentina Djordjevic ¹, Dragica Radojkovic ¹

*Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a,
P.O. Box 23, 11010 Belgrade, Serbia and Montenegro*

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Abstract

Alpha-1-antitrypsin (AAT) is a serine protease inhibitor whose deficiency could cause emphysema and liver disease and, as recently described, could be a risk factor for lung cancer development. Alpha-1-antitrypsin inhibits a variety of proteases but its primary target is neutrophil elastase, an extracellular endopeptidase capable of degrading most protein components of the extracellular matrix. Inhibition of neutrophil elastase by AAT has an important role in maintaining the integrity of connective tissue. The gene encoding for AAT spans over 12.2 kb, consists of seven exons and is highly polymorphic. Therefore several methods for mutation screening of alpha-1-antitrypsin gene have been developed. Method described here is based on denaturing gradient gel electrophoresis (DGGE). This method is highly efficient and reliable and allows rapid analysis of entire coding region of alpha-1-antitrypsin gene, including splice junction sites. Previously described DGGE based analysis of AAT gene included overnight electrophoresis of individually amplified fragments. The optimization of the method described in this paper is directed towards the shortening of the duration of electrophoresis and amplification of fragments in multiplex reaction in order to make the analysis less time-consuming and therefore more efficient. © 2006 Elsevier B.V. All rights reserved.

Keywords: Alpha-1-antitrypsin; Mutation detection; Allelic variants; PCR; DGGE

1. Introduction

Alpha-1-antitrypsin (AAT) is a member of serpin superfamily of proteins, whose deficiency is associated with emphysema and liver disease [1]. Serpins are present in a wide range of species

^{*} Corresponding author. Tel.: +381 11 3976658; fax: +381 11 3975808.

E-mail address: qwert@eunet.yu (M. Ljubic).

¹ Tel.: +381 11 3976658; fax: +381 11 3975808.

and are structurally characterized by the presence of 3 β -sheets (A, B and C) and 9 α -helices (A-I) connected by loop segment. These proteins control important intracellular and extracellular pathways such as inflammatory, complement, coagulation and fibrinolytic cascades [2].

Alpha-1-antitrypsin gene is a 12.2 kb long, containing seven exons and six introns [1]. Gene is located on chromosome 14 at q31 within the cluster of related serpin genes that spans over approximately 320 kb. The first three exons (1A–1C) are non-coding and the last four exons [2–5] encode for a 394 amino acid, 52 kDa glycoprotein that is mainly synthesized in the liver.

Primary physiological role of alpha-1-antitrypsin is the protection of lower respiratory tract tissue from destruction by neutrophil elastase [1]. Deficiency of alpha-1-antitrypsin caused by mutations in alpha-1-antitrypsin gene leads to progressive destruction of alveoli which eventually culminates in emphysema. Liver disease outcomes as a result of intracellular accumulation of particular mutant variants in hepatocytes. Recent studies have shown that deficiency of alpha-1-antitrypsin is associated with increased risk for lung cancer development [3,4]. More than 100 allelic variants of alpha-1-antitrypsin have been identified to date [5]. Based on plasma level and function of AAT these variants are categorized as normal, deficient, null and dysfunctional [6].

Standard method used for detection of alpha-1-antitrypsin deficient variants is phenotyping by isoelectric focusing (IEF) of serum proteins (pH 4.2–4.9) combined with nephelometric determination of plasma AAT concentration. This kind of diagnosis is not always accurate because both phenotypes determined by IEF and AAT serum concentration vary due to external factors such as inflammation and injury [7]. Aside from that, some mutations in AAT gene could be misinterpreted and mutations resulting in the low serum level of AAT and null variants are difficult to detect. Genotyping methods described in the past decade for analysis of AAT are either restricted to detection of specific and known mutations, such as restriction fragment length polymorphism (RFLP) method used for detection of the most common mutations (S and Z), or expensive and time-consuming, such as DNA sequencing. Since the symptoms of AAT deficiency differ due to the type of the mutation present, and that phenotyping methods are not always reliable, mutation screening of the entire gene is needed for accurate identification of variants.

Different changes in DNA sequence such as single base pair substitutions, insertions and deletions can be detected by denaturing gradient gel electrophoresis (DGGE) with high efficiency and sensitivity. This method was developed two decades ago and it represents a highly sensitive electrophoretic separation technique based on differences in melting behavior of double-stranded DNA fragments in a gradient with an increasing concentration of denaturant [8]. Fragments that differ in one or more nucleotides have different melting temperatures. Fragments run through the gel with an increasing concentration of chemical denaturants (formamid and urea) until they come to the point at which the strands with the lowest melting temperature dissociate and the gel motility of molecule is rapidly slowed. To prevent complete strand dissociation and to facilitate the detection of mutations in the higher melting domains, a GC-rich fragment (GC-clamp) is introduced during fragment amplification. The use of GC-clamped primers prevents the fragment from melting completely and therefore ensures reproducibility of the method. It also alters the melting characteristics of the fragment allowing the detection of mutations in the melted part, which increases the sensitivity of DGGE method to theoretically 100% [9].

No standard exists with regard to gel composition and electrophoretic conditions for DGGE analysis. Several factors can influence the efficiency of DGGE mutation detection, such as primer design (including the length, position and nucleotide sequence of the GC-clamp), concentration of polyacrylamide, addition of glycerol and glycerol gradients and variations in denaturing gradients.

The previously published methods [7,10] for analysis of AAT gene using DGGE included overnight electrophoresis of individually amplified DNA fragments. The objective of this work

was to improve the analysis by multiplex amplification of fragments and by shortening the time of electrophoresis.

2. Material and methods

2.1. DNA amplification

Polymerase chain reaction (PCR) amplification was performed on whole blood samples. Blood was taken with 0.129 mol/L $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ as anticoagulant. Amplifications were performed in a 50 μL reaction mixture, containing 1 \times *Taq* buffer (50 mM KCl, 10 mM Tris–HCl, pH 9, 0.1% Triton X-100), 0.2 mM of each dNTP, 2.5 mM MgCl_2 and 2 μL of blood. Optimal concentration of primers was determined for amplification of each amplicon (30 pmol of each primer for exon 2A, 30 pmol of each primer for exon 2B, 30 pmol of each primer for exon 4 and 30 pmol of each primer for exon 5B). Exons 2C, 3 and 5A were amplified in multiplex reaction containing 25 pmol of each primer for exon 2C, 35 pmol of each primer for exon 3, and 20 pmol of each primer for exon 5A. Previously designed primers [10] and amplification conditions are shown in the Table 1. The reaction mixtures were first subjected to the hot start (6 cycles of heating at 98 °C for 3 min and cooling at 55 °C for 3 min) after which 1 U of *Taq* polymerase (AmpliTaq Gold, Applied Biosystems) was added to the mixture. Amplifications were performed using following conditions: an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for exons 2B and 5B and 54 °C for exons 2A, 2C, 3, 4 and 5A for 1 min, elongation at 72 °C for 1 min and final elongation at 72 °C for 10 min. Length, purity and yield of amplified fragments were checked by electrophoresis on 2% agarose gels stained with ethidium bromide. DNA was visualized under UV light.

2.2. Denaturing gradient gel electrophoresis (DGGE)

Electrophoresis was performed in a 6.5% polyacrylamide gel (acrylamide/bisacrylamide 19:1) containing gradient of urea and formamide in a 1 \times TAE buffer (40 mM Tris, 20 mM $\text{C}_2\text{H}_3\text{O}_2\text{Na}$,

Table 1
Primer sequences and amplification conditions

Exon	Primer sequence (5'–3')	Amplicon size (bp)	Annealing temperature (°C)
2 (2A)	TCATCATGTGCCTTGACTCG (40GC) GGTATAGGCTGAAGGCGAAC	280	54
2 (2B)	(40GC) CCACCATGATCAGGATCACC TCCACTAGCTTCAGGCCCTC	382	58
2(2C)	CAATGGCCTGTTCTCTCAGC (40GC)GCCAAGGAGAGTTCAAGAACTG	357	54
3	TCTTCCAAACCTTCACTCACC (40GC) TTCTTGGTCACCCTCAGGTT	393	54
4	(40GC) GAACAAGAGGAATGCTGTGC ATGGTGCAACAAGGTCGTC	270	54
5(5A)	GCCTTACAACGTGTCTCTGC (40GC) GATAGACATGGGTATGGCCTC	162	54
5(5B)	GAAAGGGACTGAAGCTGCTG (40GC) GTTGAGGAGCGAGAGGCAG	219	58

40GC – CGCCCGCCGCGCCCGCGCCCGGCCCGCCGCCCCGCCCCG.

1 mM Na_2EDTA , pH 7.4) at 58 °C and 240 V using DENATURING GRADIENT GEL ELECTROPHORESIS SYSTEM (C.B.S. SCIENTIFIC). DNA samples were prepared for loading onto a gel by forming of heteroduplexes by denaturation at 95 °C for 5 min and subsequent incubation at annealing temperature for 5 min. DNA bands on gel were visualized by silver staining [11]. Optimization of conditions for analysis of alpha-1-antitrypsin gene using DGGE method included variation in concentration of denaturants and duration of electrophoresis.

3. Results

For the purpose of analysis of entire coding region of AAT gene including splice junction sites, exons 2 and 5 were divided into 3 amplicons for exon 2 (2A, 2B and 2C) and 2 amplicons for exon 5 (5A and 5B) because of complex melting patterns, while exons 3 and 4 were amplified in single reactions. The amplification was performed directly, on the whole blood samples. This enables

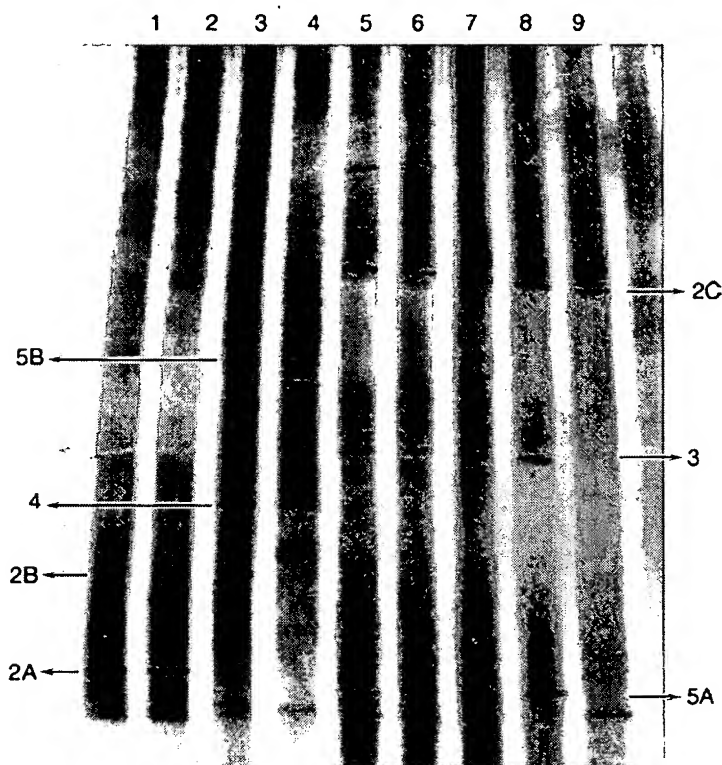


Fig. 1. DGGE band pattern of seven amplicons of alpha-1-antitrypsin gene. Lane 1: amplicon 2B – homozygous R101R; amplicon 2A – wild type pattern. Lane 2: amplicon 2B – heterozygous R101H; amplicon 2A – wild type pattern. Lane 3: amplicon 5B – homozygous D376D; amplicon 4 – wild type pattern. Lane 4: amplicon 5B – heterozygous E376D; amplicon 4 – wild type pattern. Lane 5: amplicon 2C – wild type pattern, amplicon 3 – heterozygous V213A, amplicon 5A – heterozygous E342K; Lane 6: amplicon 2C – wild type pattern, amplicon 3 – heterozygous V213A, amplicon 5A – homozygous E332E; Lane 7: amplicon 2C – wild type pattern, amplicon 3 – heterozygous V213A, amplicon 5A – heterozygous E332K; Lane 8: amplicon 2C – wild type pattern, amplicon 3 – homozygous A213A, amplicon 5A – homozygous K332K; Lane 9: amplicon 2C – wild type pattern, amplicon 3 – homozygous V213V, amplicon 5A – homozygous E332E.

use of small sample amounts and shortens time required for analysis. Analysis is additionally facilitated by simultaneous amplification of exons 2C, 3 and 5A in multiplex reaction, as well as with pooling the other four amplicons in pairs before electrophoresis.

The fragments obtained by whole blood amplification were analyzed by DGGE. Standardization of conditions for DGGE analysis included variations in concentrations of denaturants and duration of electrophoresis and it was performed on control samples previously characterized by sequencing. Electrophoresis in the gradient of denaturants from 40% to 80% (100% denaturant – 7 M urea and 40% formamide) for either 6 or 8 h showed no band separation in control heterozygous samples. Band separation for all amplicons was accomplished in 6.5% polyacrylamide gel with gradient of denaturants ranging from 20% to 70% for 6 h at 240 V and therefore was optimal for analysis of entire AAT gene (Fig. 1). Use of 6.5% polyacrylamide gel rather than gel with higher percentage of polyacrylamide in combination with high voltage enables fragments to run faster through gel and to reach optimal melting temperature earlier in the gel, so the duration of electrophoresis for 6 h is sufficient for optimal band separation.

4. Discussion

The accurate diagnosis of alpha-1-antitrypsin deficiency is critical for proper treatment of affected individuals considering that clinical features highly depend on the present mutations. The diagnosis of AAT deficiency is still mostly based on IEF of serum proteins, in combination with plasma AAT concentration measurement. However, application of IEF method can often lead to misinterpretation of some AAT gene mutations. Genotype ZZ is frequently misclassified as PiSZ phenotype [12]. Also, it has been known that IEF phenotype varies according to external factors such as inflammation, medication or injury [13].

Since conclusions based on routinely used methods, IEF of serum proteins and plasma AAT concentration measurement, can lead to misdiagnosis, the genotyping methods have inevitably evolved in order to improve diagnosis of alpha-1-antitrypsin deficiency. Although several methods for alpha-1-antitrypsin gene mutation detection have been developed to date, their part in routine diagnostics of alpha-1-antitrypsin deficiency is still relatively small. Several genotyping methods are currently applied for detection of AAT gene mutations, mostly DNA sequencing, real-time PCR and RFLP. Although their reliability makes them useful for diagnostic purposes, DNA sequencing and Real-time PCR are quite expensive and time-consuming methods and therefore not routinely applied in AAT deficiency diagnostics. Since RFLP is much more rapid and less expensive method it is more adequate and therefore more widely used for routine diagnostics. This method is usually applied to detect only the most frequent AAT gene mutations, Z and S, since they are present on more than 90% of chromosomes in AAT deficient individuals [14]. However, this method fails to detect rare AAT variants, which account for 2–4% of AAT deficient individuals [15]. Considering that more than 100 mutations have been identified to date and that they are randomly distributed in the AAT gene, methods which enable whole gene screening and give high mutation detection rate better meet the requirements of AAT deficiency diagnostics.

Denaturing gradient gel electrophoresis (DGGE) is accurate and reproducible method convenient for mutation detection. Successful application of DGGE for analysis of each DNA fragment requires optimal experimental conditions because the melting behavior of fragment is highly sequence-dependent. The efficiency of mutation detection by DGGE is influenced by primer design, gel composition and electrophoretic conditions. If conditions are optimized, the efficiency of DGGE mutation detection achieves almost 100% [9]. Due to its very high sensitivity and reliability DGGE is one of the most efficient methods for whole gene screening and it is applied for indirect diagnostics

of many hereditary diseases. Application of DGGE method offers possibility to detect not only most frequent and already known mutations, but also to detect rare and, eventually, new ones. In addition, DGGE method enables accurate and rapid molecular diagnosis at relatively low cost. Therefore, it is especially valuable for the screening of genes in which mutations are numerous and randomly distributed, such as AAT gene. While IEF phenotypization has a possibility of misdiagnosis of AAT deficiency, DGGE is highly accurate and a chance of misinterpretation practically does not exist. Direct comparison of these two methods has shown that IEF phenotyping achieves only 85% efficiency, while DGGE genotyping is highly conclusive [7]. Apart from its advantages when compared to phenotyping techniques, the whole gene screening by DGGE method is also advantageous, especially in combination with multiplex PCR, in comparison with other genotyping methods.

5. Simplified description of the method

Denaturing gradient gel electrophoresis is highly sensitive electrophoretic technique based on differences in melting behavior of double-stranded DNA fragments in a gradient with an increasing concentration of chemical denaturants.

Amplification of all four coding exons of alpha-1-antitrypsin gene including splice junction sites was performed directly on whole blood samples in one multiple and four individual reactions. The amplified fragments were analyzed by DGGE in 6.5% polyacrylamide gel with gradient of denaturants from 20% to 70% for 6 h at 240 V. The described method, based on amplification from whole blood and multiplex analysis, decreases time and costs of the procedure, which makes it optimal for application in diagnostics. Besides the detection of the most frequent and already known mutations, this method enables detection of the rare and, eventually, new ones.

Acknowledgments

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REVIEW SERIES

 α_1 -Antitrypsin deficiency · 1: Epidemiology of α_1 -antitrypsin deficiency

M Luisetti, N Seersholm

Thorax 2004;59:164-169

The protein and molecular characteristics of variants of the α_1 -antitrypsin (AAT) gene are described, and available data on the genetic epidemiology of AAT deficiency are presented.

In the last 40 years, following the publication of the seminal paper by Laurell and Eriksson,¹ there have been significant advances in the understanding of genetic abnormalities related to α_1 -antitrypsin (AAT) deficiency and of the pathophysiology of the associated lung and liver diseases. During the same period, data from a number of genetic epidemiology surveys have been accumulated. As a result, we now have a fairly comprehensive picture of the distribution of AAT deficiency, especially in developed countries, and some soundly based hypotheses about AAT gene evolution, the origin of AAT deficiency, and its spread. This paper reviews the available data on the genetic epidemiology of AAT deficiency. A preliminary discussion on the protein and molecular characteristics of AAT variants provide a background to facilitate a better understanding of the nomenclature and epidemiology data discussed.

THE AAT PROTEIN

α_1 -antitrypsin (AAT or α_1 -AT, also referred to as α_1 -proteinase (or protease) inhibitor (α_1 -PI)) is a 52 kD glycoprotein mostly secreted by hepatocytes and, to a lesser extent, by lung epithelial cells and phagocytes. It inhibits a variety of serine proteinases but its preferred target is human neutrophil elastase (HNE), for which it demonstrates the highest association rate constant.² The major function of AAT in the lungs is to protect the connective tissue from HNE released from triggered neutrophils, as supported by the development of pulmonary emphysema early in life in subjects affected by severe inherited deficiency of AAT.³ In the majority of humans the lungs are defended from HNE attack by normal AAT plasma levels ranging from 100 to 200 mg/dl (as measured by nephelometry). Although AAT is a well known acute phase reactant, this wide variability in its normal plasma levels mostly reflects the marked pleomorphism of the glycoprotein. More than 100 genetic variants of AAT have been identified and these are strictly associated with specific AAT plasma levels in a co-dominantly inherited fashion⁴—in other words, plasma AAT levels

are determined by both AAT gene alleles independently of each other.

The nomenclature currently used to identify the AAT variants is a sort of compromise resulting from the evolution of the different techniques applied to separate and characterise the proteins over the last 40 years. The AAT variants included in an allelic system called the Pi (protease inhibitor) system were initially named on the basis of their migration velocity in starch-gel electrophoresis as M (medium), S (slow), F (fast), or Z (very slow).⁴ Subsequently, when proteins began to be separated on the basis of their isoelectric point (pH 4–5 isoelectric focusing (IEF) on thin layer polyacrylamide gel), to cope with the previous nomenclature system the AAT variants were classified with the first letters of the alphabet if displaying anodal migration and with the last letters if displaying cathodal migration. At the advent of the genomic era the former Pi system was renamed PI* to identify the AAT gene locus.⁵

After the original paper by Laurell and Eriksson¹ and the subsequent evidence that most subjects with inherited severe deficiency of AAT were predisposed to an early onset of emphysema,⁷ it became useful for clinical purposes to classify AAT variants into three major categories:⁴

- Normal, characterised by AAT plasma levels within general population reference ranges, not associated with a risk of lung or liver disease. This category includes the four most common middle migrating M variants (M1→M4) and a number of less common variants identified on the basis of the alphabet letter, as indicated above, and the city of the oldest living carrier of the variant⁸—for example, L_{Frankfurt}.
- Deficient, characterised by reduced but detectable AAT plasma levels, associated with an increased risk of developing lung or liver disease. This category includes the most frequent deficient variants, Z and S, and a number of less frequent variants including the so called M-like variants (M_{malton}, M_{prociada}, etc), with a middle migrating pattern. The upper limit of the plasma AAT level to include an AAT variant in this category is 80 mg/dl—that is, that displayed by most subjects with the PI*SZ genotype.
- Null (currently designated QO), with no detectable plasma AAT level, associated with an increased risk of developing emphysema.

See end of article for authors' affiliations

Correspondence to:
Dr M Luisetti, Clinica di
Malattie dell'Apparato
Respiratorio, IRCCS
Policlinico San Matteo, Via
Taramelli 5, 27100 Pavia,
Italy;
m.luisetti@smatteo.pv.it

THE AAT GENE AND ITS EVOLUTION

Knowledge of the molecular structure of the AAT gene began to emerge roughly two decades after the report of the serum protein deficiency.⁹ The AAT gene is part of a gene cluster, located on human chromosome 14q32.1, called the SERPIN (SERine Proteinase INhibitor) supergene. This gene cluster includes the corticosteroid binding globulin (CBG), AAT-like pseudogene (PIL), AAT, protein C inhibitor (PCI), and α_1 -antichymotrypsin (AACT) genes (centromere to telomere). The AAT gene spans 12.2 kb in length and has three non-coding (IA, IB, IC) and four coding (II, III, IV, V) exons; exon V contains the sequence coding for the reactive site of the AAT protein (Met³⁵⁸-Ser³⁵⁹). There is a close genetic linkage between the AAT and AACT genes, and it is likely that the two loci differentiated relatively recently (100–250 million years ago).¹⁰ Only a few nucleotide differences have been detected between AAT in higher primates (baboons, gorillas, and chimpanzees) and the human AAT ancestral variant, all displaying the Arg¹⁰¹-Ala²¹³-Glu³⁷⁶ combination.¹¹ Based on substitutions of these three major amino acids as haplotype markers, it has been possible to draw a likely phylogenetic tree of the major normal AAT variants and of the most frequently detected deficient variants (fig 1).

STUDIES ON THE DISTRIBUTION OF AAT DEFICIENCY

Although, as discussed below, a huge number of cohorts have been investigated, there have been only a few population based studies on AAT allele frequencies, mostly based on blood donor screening. Two seminal papers have recently reviewed the available data on the geographical distribution of AAT deficiency.^{12, 13} Given the widespread acceptance that the disorder arose in European populations, it was logical for Hutchison to address his paper on the distribution of AAT deficiency in Europe.¹² However, in the light of growing awareness of genetic conditions, de Serres¹³ recently enlarged the previous analysis to a summary of worldwide surveys, also looking at racial/ethnic differences in the prevalence of AAT deficiency. Both reviews drew on published genetic epidemiology surveys and a huge number of studies (373 control cohorts in de Serres' paper¹³) so that, for the first

time, we have a global—if not yet comprehensive—view of AAT deficiency. Of course, both reviews have the limitations inherent in meta-analyses of studies that differ in several aspects.¹²

Selection of cohorts

The surveys include a wide variety of subjects: blood donors, neonates, pregnant women, various groups of workers, students, and subjects submitted to parentage tests, laboratory or hospital staff, or "randomly" selected individuals. In many instances no details about selection criteria are available.

Sample size

The survey sample sizes vary from a few dozen to several thousands of subjects, with a high percentage of surveys including 100–500 subjects. Gene frequency estimates from cohorts of fewer than 200 individuals have a high risk of error.

Methods of ascertainment of AAT variants

In most of the studies the AAT variants were determined by IEF (also referred to as "phenotyping"), although crossed immunoelectrophoresis was used in many of the older surveys published before the 1980s. The two methods are not completely interchangeable.⁵ To date, no genetic epidemiology surveys on AAT deficiency using molecular methods ("genotyping") have been reported. Given these differences, some degree of bias may have been introduced into the global analysis of the surveys.

Analysis of the genetic epidemiology surveys

The prevalence of the three major AAT variants (PI*M, PI*Z, and PI*S) is reported in most surveys as *gene frequencies*—that is, the frequency of a variant in homozygotes (where the variant contributes two alleles) or in heterozygotes (where the variant contributes one allele), and quoted as 0.0 ... *n* (or *n* per 1000 individuals). A step beyond is to use gene frequencies with the Hardy-Weinberg equilibrium formula to estimate the total number of carriers (PI*MS and PI*MZ) and subjects with deficiency variant combinations (PI*SS, PI*SZ, and PI*ZZ). This approach was used by de Serres¹³ to estimate the population at risk (carriers + subjects with deficiency

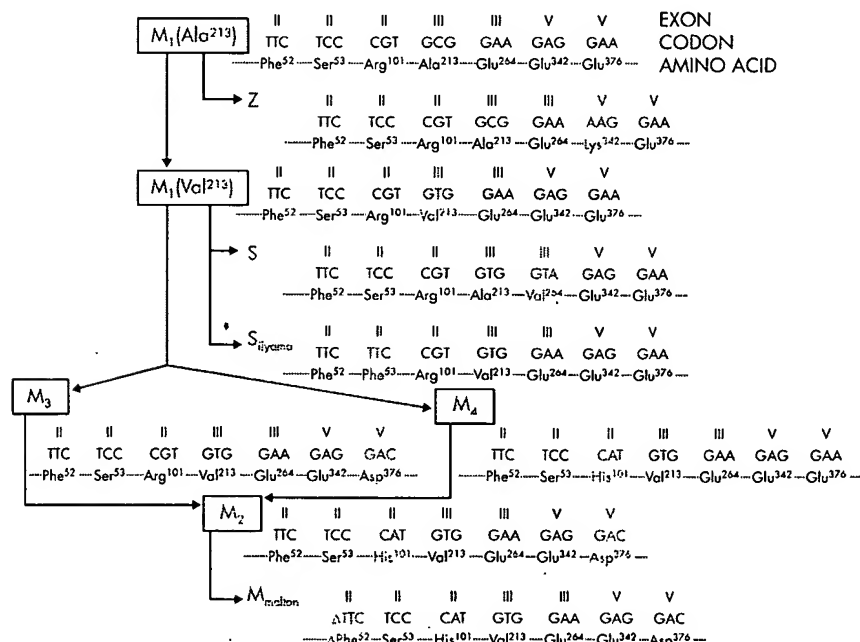


Figure 1 Phylogenetic tree of the AAT gene. Modified from Nukiwa *et al.*¹¹

variants) in a given country or geographical region, taking into account the overall population.

GENETIC EPIDEMIOLOGY OF AAT DEFICIENCY

Europe

The highest prevalence of the PI*Z variant was recorded in northern and western European countries (mean gene frequency 0.0140),¹³ peaking in southern Scandinavia, Denmark, the Netherlands, the UK, and northern France (gene frequency >0.0200).^{14–20} The results of mass screening of the whole population of neonates in Sweden performed over a 2 year period were published in 1976.²¹ Of the 200 000 infants screened, 129 had the PI*Z variant, yielding a frequency of 1 in 1550 individuals and a gene frequency of 0.026. Sveger also screened 11 000 healthy 18 year old men and found five PI*Z and 10 PI*SZ individuals.²² The most recently published study on the subject is that from the Copenhagen City Heart Study in which 9187 randomly selected subjects were investigated.²³ The prevalence found in this study (1 in 1500 individuals) is the same as that in the Swedish study,²¹ but the PI*Z gene frequency was found to be 0.049. The prevalence of PI*Z gradually decreases throughout European countries in a north-west → south-east direction, the lowest figures being recorded in eastern Europe.¹²

The distribution of PI*S differs markedly from that of PI*Z and is more homogeneous.²⁴ The highest frequency of PI*S is in southern Europe (mean gene frequency 0.0564),¹³ peaking in the Iberian peninsula (gene frequency >0.1400).^{25–26} The distribution of PI*S gradually decreases along a south-west → north-east gradient. The distributions of both PI*Z and PI*S in Europe are summarised in fig 2.

The mean ratios of PI*S:PI*Z are 4.5:1 in southern Europe, 3.5:1 in western Europe, and 1.1:1 in northern Europe (calculated from de Serres *et al*¹³).

Genetic epidemiology of AAT deficiency in particular European populations

As stated above, Scandinavia is one of the European regions with the highest figures for the PI*Z type; nevertheless, gene frequencies for both PI*S and PI*Z among Finnish and Swedish Lapps are at the lowest end of the European frequencies.^{27–28}

Analysis of 40 cohorts from Italy (reviewed by de Serres *et al*²⁹) showed that the gene frequencies of PI*S and PI*Z are highest in northern Italy and decrease gradually from north to south. In one of the few available population based surveys performed in 9000 neonates in South Tyrol, a comparison between German and Italian individuals yielded a PI*Z gene frequency of 0.019 and 0.015, respectively.³⁰ In Sardinians the gene frequency of PI*S is higher than in continental Italy whereas that of PI*Z is much lower.

The frequency of PI*S in the Basque region is as high as in the rest of the Iberian peninsula, whereas the frequency of the PI*Z type is much lower.^{31–32}

Although one could suppose that, at least for the Lapps, susceptibility genes for pulmonary diseases such as PI*S and PI*Z may have been eliminated by the unfavourable climate, a more likely explanation for the diversity in PI* gene frequencies lies in the great isolation of the Lapp, Sardinian, and Basque populations from other genetic influences. In fact, analysis of major histocompatibility complex class I alleles revealed that these populations have marked genetic differences from other surrounding populations.³³

Other developed countries

North America

Since it is widely accepted that AAT deficiency arose in European populations, the spread of the disorder in countries whose inhabitants have a European background is not

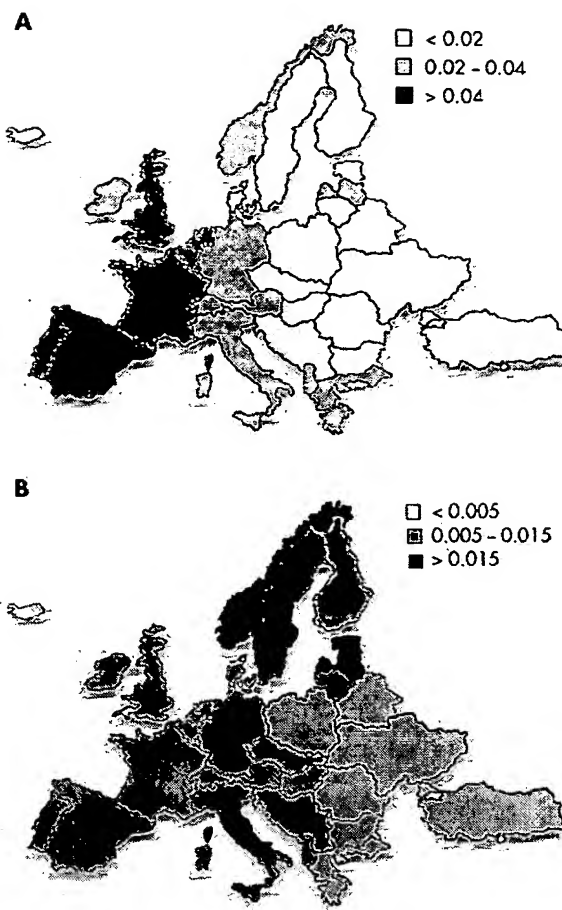


Figure 2 Frequencies of (A) PI*S and (B) PI*Z genes in Europe.

surprising.³⁴ However, the average gene frequency of PI*Z in North America is 0.0092 (at the lowest end of the range reported in Europe), whereas the frequency of the PI*S gene is 0.0328 which is higher than that reported for Northern Europe.¹³ This figure might be due to very mixed populations in North America and to the limited number of cohorts available (43) with respect to the overall population. A population based survey performed in 20 000 blood donors in the St Louis area yielded a PI*Z prevalence of 1 in 2800 individuals.³⁵

Australia and New Zealand

The gene frequencies of PI*Z and PI*S in Australia and New Zealand are very close to those reported for North America (0.0151 and 0.0395, respectively), probably for the same reasons.

Far East Asia

Very limited cohorts have been reported from Japan, China, and South Korea.¹³ The gene frequency of PI*Z is 0.0002 in Japan, 0 in China, and 0.0061 in South Korea, whereas the respective figures for PI*S are 0.0004, 0.0006, and 0.0070. Interestingly, the AAT M1 (Ala²¹³) variant, found in approximately 20–23% of AAT deficient white subjects,¹¹ was not detected in any of 156 Japanese subjects. Since the Z variant has developed on the M1 (Ala²¹³) base allele (fig 1), this may account for the extreme rarity of the PI*Z gene in the Japanese and other Far Eastern populations. These

findings also account for the prevalence of the Siiyama deficiency variant in the Japanese; this variant arose on the M1 (Val 213) base allele (fig 1)^{36, 37} and was present in 100% of the 156 Japanese investigated.

South America

Only a few cohorts from Southern America have been reported, so no firm data can be presented.¹³

Developing countries

The belief that AAT deficiency is a disorder which mostly affects white subjects has been, in part, shaken by the analysis of the worldwide surveys performed by de Serres.¹³ He provided evidence for a significant prevalence of both PI*Z and PI*S in populations from the Middle East and North Africa, Central and Southern Africa, and Central and South-East Asia, suggesting that AAT deficiency has prevailed over racial and ethnic boundaries.

AGE ESTIMATES OF AAT DEFICIENT VARIANTS AND THEIR DIFFUSION THROUGH POPULATIONS

The analysis of allelic variants within the serpin gene cluster in defined populations may yield useful information about the time and site of origin of AAT deficient variants. In an investigation of white PI*Z families of northern European origin, Byth and coworkers³⁸ found that 97% of cases had a unique haplotype of 60 kB encompassing the CBG, PIL, and AAT genes associated with the PI*Z allele, thus supporting the theory of a single origin for the PI*Z mutation.³⁹ Haplotype analysis also allows an estimate of the time the PI*Z mutation first occurred. Based on the assumption of random recombination in a given area, Byth hypothesised that the PI*Z mutation might have arisen 66 generations ago—that is, assuming 33 years to be the mean lag for each generation, ~2000 years ago. This estimate differs from a previous hypothesis of 216 generations (~7000 years ago)³⁹ and a more recently advanced estimate of 120 generations (~4000 years ago⁴⁰). Interestingly, according to the last estimate, the PI*Z mutation could have been dispersed during the Neolithic era, as has been suggested for the cystic fibrosis $\Delta F508$ mutation.^{40, 41} According to the hypothesis that the higher the gene frequency in a given country, the more likely it is that the gene first occurred there,¹² it is commonly accepted that the PI*Z gene arose in northern Europe (and maybe more precisely in southern Scandinavia) and subsequently spread to other European countries and to countries bordering the Mediterranean Sea, following the known major population movements in Europe such as the Viking voyages. Nevertheless, the facts that patterns of haplotype diversity

contrast with the expected stepwise reduction if the mutation spread from north to south, as demonstrated in populations from the Iberian peninsula,⁴⁰ and that PI*Z types are found in populations from Central and South Africa and from Asia,¹³ suggest a diffusion against the main known directions of population movements or, alternatively, a multiregional origin for the PI*Z gene.

The finding that the prevalence of PI*S is highest in the Iberian peninsula indicates that the PI*S gene probably originated in this area, and perhaps more precisely in the Portuguese population.⁴⁰ Interestingly, serpin haplotype investigation in this population suggests that this event occurred 15 000–10 000 years (450–300 generations) ago, making the PI*S mutation much older than the PI*Z one. At variance with the European spread of PI*Z, the west to east gradient of PI*S mutation indicates a diffusion against the known major population movements in Europe.

WORLDWIDE ESTIMATES OF SUBJECTS WITH AAT DEFICIENCY

Taking into account the gene frequencies of PI*S and PI*Z reported in the genetic epidemiology surveys retrieved from the international literature and the number of individuals in the total populations in different countries, de Serres¹³ calculated worldwide estimates of subjects affected by intermediate AAT deficiency (that is, carriers) and of subjects at high risk of developing lung/liver disease associated with AAT deficiency (that is, PI*Z homozygotes and PI*SZ compound heterozygotes). Of course, such an approach incorporates potential biases: in addition to the above reported limitations of the studies considered, some investigations were performed in selected populations which were poorly representative of the general population.²⁹ As an example, the cohorts examined for Italy included a number from the valleys on the south side of the Alps³⁰ (where geological barriers are evident, separating these cohorts from those recruited among inhabitants of the nearby plains), and from Sardinia (whose genetic isolation has been already discussed³¹). These results should therefore be considered with caution. Despite these caveats, the overall estimates of approximately 116 000 000 carriers and 1 100 000 subjects with severe AAT deficiency worldwide are astonishing and indicate that AAT deficiency is probably one of the most common severe hereditary disorders in the world (table 1).

OPEN QUESTIONS AND FUTURE DIRECTIONS

AAT deficiency is an under-recognised condition

Taking into account the above mentioned estimates, it is evident—not only to physicians actively involved in the

Table 1 Estimates of the worldwide numbers of carriers (Pi MS and PiMZ) and subjects at high risk for developing lung/liver disease associated with AAT deficiency (excluding Central and South America)

Geographical region	Carriers		Individuals with AAT deficiency	
	Pi MS	Pi MZ	Pi SZ	Pi ZZ
Northern Europe	1064350	1027452	21150	11578
Central Europe	10499896	3933048	85661	17514
Southern Europe	20148269	3946672	262780	27515
Western Europe	5337818	1495680	71983	10146
North America	18469434	7155901	257708	53173
Australia/New Zealand	1816658	639174	28231	5476
Middle East/North Africa	1669090	903232	32266	10657
Africa	17334307	1404344	75096	6412
Central Asia	6499962	4506979	40815	20504
South-east Asia	4063472	1605298	37898	10706
Far East Asia	1911276	607460	3553	1771
Total	88814533	27225242	929014	175268

Simplified from de Serres.¹³

Table 2 Relationship between expected and diagnosed cases of AAT deficiency (Pi ZZ + Pi SZ) in selected countries

Country	AAT deficiency expected	AAT deficiency diagnosed
Canada	42372	144
Italy	46068	100
The Netherlands	9790	136
New Zealand/Australia	33707	93
Spain	86899	90
Sweden	6717	181
UK	79456	324
Total	305009	1068

Expected cases are based on de Serres¹³ and Martin *et al*²⁶; diagnosed cases are from Alpha One International Registry (AIR) central database (updated April 2003, courtesy of Claes-Göran Löfdahl, Eeva Piitulainen, Ragnar Alm). Individuals with AAT deficiency in the AIR database were recruited since 1999 in a prospective fashion.

diagnosis and management of AAT deficiency—that this is a largely under-recognised condition. The availability of AAT replacement therapy for individuals with pulmonary emphysema associated with AAT deficiency⁴² encouraged the scientific community to establish and reinforce AAT deficiency screening programmes in developed countries, even in those not previously considered to have a high prevalence of the disorder, and to implement national registries.⁴³ In response to a suggestion forwarded during a WHO meeting on AAT deficiency,⁴⁴ an international registry confederating national registries from several countries was established in 1996.^{44–45} In spite of the extensive efforts made to identify cases with AAT deficiency, it is clear that only a small minority of subjects are actually recognised (table 2).

There are at least two reasons for this under-recognition. Firstly, the clinical phenotypes associated with AAT deficiency (pulmonary emphysema, chronic bronchitis, bronchiectasis, asthma and, to a lesser extent, chronic liver disease) are not exclusive to the condition. Even familial aggregation of the phenotype, a typical feature of inherited disorders, is not a useful sign since common chronic obstructive pulmonary disease often tends to cluster in families.⁴⁶ Secondly, the PI*Z gene is characterised by an incomplete penetrance—that is, the relationship between genotype and clinical phenotype is not strong. Silverman and coworkers examined pulmonary function in a cohort of 52 PI*Z subjects: 20 out of the 52 subjects (38%) had a forced expiratory volume in 1 second (FEV₁) over 65% predicted and frequently within the normal range.⁴⁷ These PI*Z subjects with normal or only mildly impaired lung function are usually identified as non-index cases—that is, cases ascertained during family screening. The same authors also found that the severity of the disease manifestations is affected by some variables such as cigarette smoking and lower respiratory tract infections (gene × environment interaction). The percentage of subjects with asymptomatic or mild AAT deficiency was even higher among a series of 94 individuals with PI*SZ compound heterozygosity.⁴⁸ In conclusion, there is evidence that many individuals with severe AAT deficiency do not have clinically significant lung function impairment. This feature of AAT deficiency deserves further investigation, both from the epidemiological and genetic viewpoints.

Epidemiology of rare (non-Z, non-S) AAT deficient variants

Little is known about the genetic epidemiology of rare AAT deficient variants which are considered not to exceed 2–4% of all variants.⁴⁹ However, the prevalence of these variants may be higher than was previously believed because rare AAT deficient variants can be mistaken for the PI*Z variant and

therefore misdiagnosed. Indeed, we have preliminary data from the Italian Registry for AAT Deficiency to indicate that as many as 22% of the total AAT deficient variants are rare.⁵⁰ The nomenclature of some of these variants (see above) reflects their probable Italian origin (M_{proclida}, M_{palermo}, QO_{isola di procida}, QO_{trastevere}).⁵¹ An intriguing question is: are the rare AAT deficient variants more frequent in those countries in which the gene frequency of PiZ is lower? Data from the island of Sardinia seem to support this hypothesis.⁵¹ Clinical phenotypes associated with the common AAT deficient variant PI*Z are reasonably well defined, as will be discussed later in this review series, but no information is so far available on clinical phenotypes associated with rare AAT deficient variants. This should be addressed by future studies.

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Authors' affiliations

M Luisetti, Laboratorio di Biochimica e Genetica, Clinica Malattie Apparato Respiratorio, IRCCS Policlinico San Matteo, Università di Pavia, Italy

N Seersholm, Department of Internal Medicine I, Bispebjerg Hospital, Copenhagen, Denmark

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α 1-ANTITRYPSIN DEFICIENCY: AN OVERVIEW

W. van Steenbergen*

SUMMARY

1. α 1-antitrypsin is an antiprotease that inhibits the
neutrophil elastase enzyme, and belongs to a family of
structurally related serine proteinase inhibitors
(serpins). Its methionine³⁵⁸ residue determines the
specificity for elastase.

2. The normal M-type α 1-antitrypsin is mainly
synthesized in the liver parenchymal cells and trans-
ported to the plasma. Abnormal Z-mutant α 1-
antitrypsin is retained in the endoplasmic reticulum,
which leads to its intracellular accumulation and to
markedly decreased plasma levels.

3. In normal conditions, α 1-antitrypsin protects the
lungs from destruction by the proteolytic neutrophil
elastase. A protease/antiprotease imbalance in the
lung is responsible for the development of emphysema
in severe α 1-antitrypsin deficiency and in cigarette
smokers, and accounts for the marked acceleration of
the lung disease in smoking α 1-antitrypsin deficient
patients. Smoking has to be avoided in α 1-antitrypsin
deficient patients. Replacement therapy with plasma-
derived α 1-antitrypsin seems indicated in α 1-
antitrypsin deficient patients with emphysema.

4. Intracellular accumulation of abnormal Z- α 1-
antitrypsin molecules in liver parenchymal cells may
lead to liver disease, ranging from neonatal cholestasis
to adulthood cirrhosis and hepatocellular carcinoma.

End-stage liver disease can be treated by liver trans-
plantation, which is followed by a phenotypic conver-
sion.

5. Diagnosis of α 1-antitrypsin deficiency related
disease relies on the presence of a low serum concen-
tration of α 1-antitrypsin, and of periodic-acid Schiff
positive globules in the liver parenchymal cells.
Isoelectric focusing of the serum identifies the protease
inhibitor phenotype. The protease inhibitor phenotype
is determined by the independent expression of the
two parental α 1-antitrypsin alleles. It is determinant of
the serum level and of the risk for development of lung
or liver disease.

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INTRODUCTION

The story of α 1-antitrypsin and its deficiency is
a nice example of the complex interdependence
of different organ systems. α 1-antitrypsin (α 1AT)
is mainly produced by the liver. Its primary
function is to protect a second organ, the lung,
from the neutrophil elastase enzyme which is
carried by cells derived from a third organ, the
bone marrow. In α 1-antitrypsin deficiency the
first organ can no longer protect the second organ
from the third, which results in early onset
emphysema. Moreover, a number of deficient
individuals develop a spectrum of liver diseases
ranging from neonatal cholestasis to adulthood
cirrhosis and hepatocellular carcinoma. This
review will deal with the normal form, synthesis,
and function of α 1AT, with the α 1AT-gene and its
alleles, with clinical manifestations of the defi-
ciency state, and with some of the current
therapeutic modalities.

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*Dienst voor Leverziekten
(Afdeling Inwendige Ziekten
UZ Gasthuisberg, Herestraat 49
B-3000 Leuven, België)

STRUCTURE AND SYNTHESIS OF α 1-ANTITRYPSIN

α 1-antitrypsin is a small 52-kDa glycoprotein consisting of a polypeptide chain with 394 aminoacids and of three asparaginase-linked carbohydrate side chains. There are two major isoforms in the serum, depending on the presence of a bi- or triantennary configuration of the carbohydrate side chains (1, 2). Inhibition of the neutrophil elastase enzyme is the major physiological function of α 1AT. The reactive site of the α 1AT molecule - the so-called PI residue - is centered on the aminoacid residues Met³⁵⁸-Ser³⁵⁹, and is part of a highly stressed, external loop protruding from the molecule (Fig. 1). This Met³⁵⁸-Ser³⁵⁹ tip fits closely into the reactive pocket of neutrophil elastase (Fig. 1). A tight interaction occurs between the inhibitor and the neutrophil elastase reactive pocket, and the elastase is prevented from functioning (2, 3). α 1-antitrypsin belongs to a family of structurally related «serpins» or serine proteinase inhibitors. These molecules function either as suicide inhibitors by forming an equimolar complex with specific target proteases or, less commonly, as binding proteins (Table 1) (4-7). The PI

residue is the most important determinant of functional specificity for each serpin molecule. For instance, α 2-antiplasmin inactivates plasmin by the interaction between its Arg³⁶⁴-Met³⁶⁵ peptide bond and the protease molecule. In the α 1AT Pittsburgh variant, the Met³⁵⁸ residue is replaced by Arg³⁵⁸. In this variant, α 1AT func-

TABLE 1: α 1-ANTITRYPSIN IS PART OF A FAMILY OF STRUCTURALLY RELATED SERPIN MOLECULES WHICH ACT AS INHIBITORS OF A SPECIFIC TARGET PROTEASE OR AS BINDING PROTEINS.

Serpins
α 1-antitrypsin
α 1-antichymotrypsin
α 2-antiplasmin
Antithrombin III
C ₁ inhibitor
Protein C inhibitor
Heparin cofactor II
Plasminogen activator inhibitor
Ovalbumin
Angiotensinogen
Corticosteroid binding globulin
Thyroid binding globulin

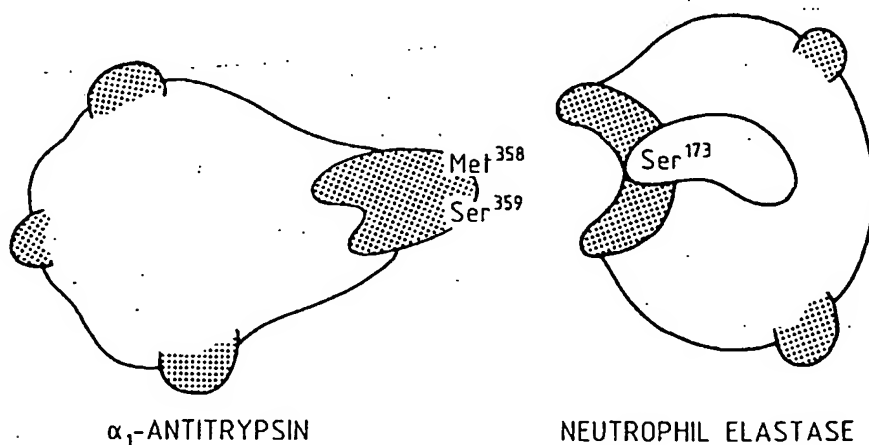


Fig. 1: The reactive site of α 1-antitrypsin, centred at the Met³⁵⁸-Ser³⁵⁹ tip, interacts with the reactive pocket of neutrophil elastase.

Acta Clinica Belgica 48.3 (1993)

tions as an inhibitor of thrombin, a severe bleeding diathesis (8). of exposure of the reactive centre loop is that it renders the molecule inactivation, either by cleavage or oxidation of the reactive Met³⁵⁸ sulfoxide. The latter derivative readily fit the reactive centre of

α 1-antitrypsin is the most abundant protease in human serum (4). In the levels of α 1AT are between 1-3 g/dl. However, these values are assessed against commercial standards used for clinical purposes, which may overestimate α 1AT levels by approximately 2-3 fold (2, 9). The true range is 20 to 48 g/dl assayed with the commercial standards. A deficiency state is defined as less than 1 g/dl, whereas the Z-homozygote level is below 50 mg/dl (2). The diagnosis of α 1AT deficiency can readily be made by serum protein electrophoresis showing a marked reduction in the alpha₁ band (Fig. 2) (11), and can be confirmed by

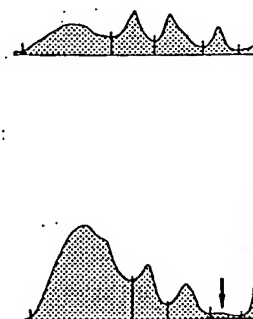
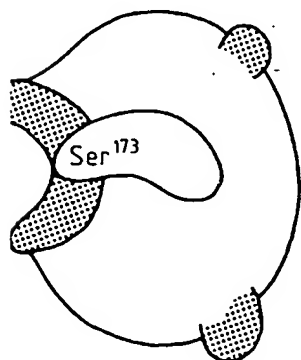


Fig. 2: Inspection of a serum protein electrophoresis strip allows the diagnosis of α 1-antitrypsin deficiency. Notice the absence of the α 1-globulin band in a patient with α 1AT deficiency.

the most important determinant of specificity for each serpin molecule. α_2 -antiplasmin inactivates plasmin by reaction between its Arg³⁶⁴-Met³⁶⁵ loop and the protease molecule. In the normal variant, the Met³⁵⁸ residue is Arg³⁵⁸. In this variant, α 1AT func-

α 1-ANTITRYPSIN IS PART OF A STRUCTURALLY RELATED SERPIN WHICH ACTS AS INHIBITORS OF A TARGET PROTEASE OR AS BINDING

trypsin
in
III
inhibitor
for II
activator inhibitor
gen
binding globulin
ing globulin



EUTROPHIL ELASTASE

ip, interacts with the reactive pocket of

tions as an inhibitor of thrombin, which results in a severe bleeding diathesis (8). A disadvantage of exposure of the reactive centre on the stressed loop is that it renders the molecule vulnerable to inactivation, either by cleavage of the loop or by oxidation of the reactive Met³⁵⁸ to methionine sulfoxide. The latter derivative is too large to readily fit the reactive centre of elastase (3, 4).

α 1-antitrypsin is the most abundant of the antiproteases in human serum (4). In normal persons, the levels of α 1AT are between 150 and 350 mg/dl. However, these values are assessed with commercial standards used for clinical studies and overestimate α 1AT levels by approximately 40% (2, 9). The true range is 20 to 48 μ M (2, 10). When assayed with the commercial standard, α 1AT deficiency state is defined as less than 80 mg/dl, whereas the Z-homozygote level is invariably below 50 mg/dl (2). The diagnosis of α 1AT deficiency can readily be made by inspection of a serum protein electrophoresis, which shows a marked reduction in the alpha-1-globulin band (Fig. 2) (11), and can be confirmed by the

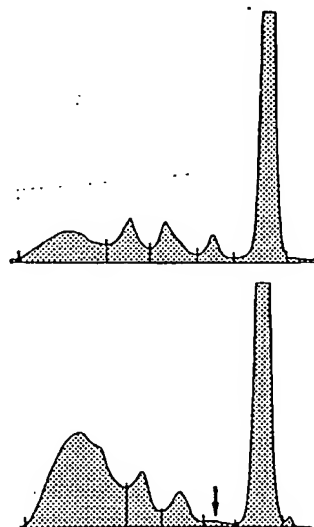


Fig. 2: Inspection of a serum protein electrophoresis strip allows the diagnosis of α 1-antitrypsin deficiency. Notice the absence of the α 1-globulin fraction (arrow) in a patient with α 1AT deficiency.

commercially available radial immunodiffusion kit (2, 9, 10). As an acute-phase reactive protein, the serum α 1AT concentration may increase considerably in response to inflammation or injury (1). Interleukin-6 is likely to be the physiological mediator of this acute-phase response (5, 12). Plasma concentrations of α 1AT also increase in patients with different types of liver disease (13), during oral contraceptive therapy and pregnancy, and following the administration of the synthetic androgen danazol (14).

The predominant site of production of plasma α 1AT is the liver. The pathway of α 1AT biosynthesis is typical of a secretory glycoprotein (2, 3). The α 1AT mRNA is translated on ribosomes bound to the rough endoplasmic reticulum. Within the cisterna of the rough endoplasmic reticulum, the three carbohydrate side chains are added as the protein begins to fold into its three-dimensional configuration. At this stage, the carbohydrate side chains are of the «high mannose» or «immature» type. Within the Golgi apparatus, further modifications of the carbohydrate side chains yield a mature α 1AT glycoprotein with «complex» type carbohydrate side chains. This mature α 1AT is secreted into the blood. During transport along this pathway, secretory proteins undergo a series of interactions with the so-called polypeptide chain-binding proteins (PCBP), which belong to the family of heat shock/stress proteins. These interactions facilitate the assembly and folding of the secretory proteins (5, 15). Once the assembly and folding of the glycoprotein is completed, secretory proteins dissociate from PCBP to allow for subsequent transport. Misfolded proteins, however, do not dissociate from PCBP. They are selectively retained in the endoplasmic reticulum until they are degraded (5, 6, 15). Binding of the abnormally folded α 1AT-Z protein by PCBP could represent the mechanism of retention of Z-proteins in the liver parenchymal cells (5, 6).

α 1-antitrypsin is also synthesized in blood monocytes and bronchoalveolar and breast milk macrophages (5, 6). Expression of α 1AT in these

Acta Clinica Belgica 48.3 (1993)

cells is markedly stimulated by interleukin 6 and by bacterial lipopolysaccharide which are generated during inflammation (5, 6, 12). The cellular defect in Z-homozygous α 1AT deficiency is also expressed in monocytes and macrophages from deficient individuals (16, 17).

α 1-antitrypsin gene expression in liver parenchymal cells, monocytes, and macrophages is regulated by a recently described feedback mechanism whereby neutrophil elastase directly regulates the synthesis of its inhibitor. Elastase- α 1AT complex binds specifically to «serpin-enzyme complex» receptors which are located on the cell surface of hepatocytes and macrophages (18). The receptor-mediated recognition of α 1AT-elastase complex leads to intracellular catabolism of the complex and to upregulation of α 1AT gene expression (19, 20).

Immunohistochemical observations and studies in transgenic mice that express the normal human α 1AT gene (21, 22) support the possibility of α 1AT gene expression in a variety of other tissues such as gastrointestinal tract, pancreas, kidney, skin, neural tissue, cartilage, and testes. Production of protease inhibitors in these organs may exert a local protective function against attack by proteases. Furthermore, these sites of expression correlate with various pathological conditions such as glomerulonephritis, pancreatitis, islet cell hyperplasia, gastric ulcer, panniculitis, arthritis, and cerebral hemorrhage, which have been described in patients with α 1AT deficiency (21, 22). It is tempting to speculate that a decreased local production of α 1AT in different organs might have a pathogenic relationship with these various disorders.

FUNCTION AND DYSFUNCTION OF α 1-ANTITRYPSIN

With a molecular mass of 52-kDa, α 1AT diffuses into most tissues and organs. Sampling of the epithelial lining fluid of the lower respiratory tract of normal persons has demonstrated average α 1AT levels of 3 to 4 μ M, which represents about

10% of normal serum levels (9, 10, 23). In the normal lower respiratory tract, α 1AT constitutes more than 80% of the anti-elastase activity (10, 24, 25). The neutrophil elastase is capable of cleaving a wide variety of components of the extracellular matrix, including elastin, the macromolecule that provides elastic recoil to the alveolar walls of the lower respiratory tract (3). Patients with α 1-antitrypsin deficiency have little or no α 1AT in their lower respiratory tract. In this situation, the anti-elastase screen is insufficient to protect the lung against the destructive capabilities of neutrophil elastase (3, 7, 10, 23-25). This protease/antiprotease imbalance slowly leads to a loss of lung elasticity, resulting in early-onset emphysema.

Theoretically, protease/antiprotease imbalance may result from failure of protease inhibition, as it is the case in α 1AT deficiency, or from excess release of elastase. This protease/antiprotease imbalance theory also explains the development of emphysema in cigarette smokers and the marked acceleration of the lung disease in α 1AT deficient patients who smoke cigarettes (4, 7, 25-28). On the one hand, smokers have evidence for a decrease in α 1AT activity in the lung (29). Inactivation of α 1AT is due to oxidation of the Met³⁵⁸ residue to methionine sulfoxide by oxidizing agents present in cigarette smoke (27, 30-32) and by oxygen radicals which are released by alveolar macrophages and polymorphonuclear leucocytes in the lungs (26, 27, 33). Exposure of the lungs to tobacco smoke causes a considerable increase in the population of pulmonary alveolar macrophages in the lung (7, 26, 27). These macrophages secrete a chemotactic factor that attracts leucocytes to the lung (26, 27, 34). Moreover, nicotine itself partly accounts for the pulmonary leucocyte recruitment observed in smokers (27). On the other hand, the smoking-induced increase in alveolar macrophages and in polymorphonuclear leucocytes leads to a large increase in the elastolytic load to the lungs (7, 26, 34, 35). A systemic increase in elastase activity has been demonstrated as well (36). According to the «two

hit concept», smoking leads both an increase in elastase and a decreased activity of the α 1-antitrypsin (25, 34). Cigarette smokers have a common centrilobular type of emphysema, whereas in α 1AT-deficient patients are predominant basal panlobular type of emphysema. In this condition, lung damage is caused by elastase released from neutrophils which enter the circulation blood. Neutrophils are sequestered at the bases because of decreased blood flow to that part of the lung. From these data, it is clear that cigarette smokers with α 1-antitrypsin deficiency are at a higher risk to develop lung disease. It has been shown that the median age at onset of dyspnea in α 1AT-deficient patients was 45 years, whereas in non-smoking deficient patients was 55 years. Moreover, life expectancy was significantly longer in non-smoking deficient patients (39).

TABLE 2. CLASSIFICATION OF α 1-ANTITRYPSIN DEFICIENCY

Category	Variant name
Normal	M ₁ (Val ²¹³) M ₁ (Ala ²¹³) M ₂ M ₃ M ₄ Others
Deficient	Z S M ^{Heerlen} M ^{Procida} M ^{Malton} M ^{Duarte} Others
Null	Null ^{granite falls} Null ^{bellingham} Null ^{matawa} Others
Dysfunctional	Pittsburgh

^aFor Caucasians; ^bRare variant

nal serum levels (9, 10, 23). In the respiratory tract, α 1AT constitutes 10% of the anti-elastase activity (10). Neutrophil elastase is capable of a wide variety of components of the extracellular matrix, including elastin, the molecule that provides elastic recoil to the walls of the lower respiratory tract (3). α 1-antitrypsin deficiency has little effect on the lower respiratory tract. In this case, the anti-elastase screen is insufficient to protect the lung against the destructive effect of neutrophil elastase (3, 7, 10, 23). The protease/antiprotease imbalance slowly causes loss of lung elasticity, resulting in emphysema.

Secondly, protease/antiprotease imbalance from failure of protease inhibition, as in α 1AT deficiency, or from excess elastase. This protease/antiprotease theory also explains the development of emphysema in cigarette smokers and the acceleration of the lung disease in α 1AT deficient patients who smoke cigarettes (4, 7, 25). On the one hand, smokers have evidence for low α 1AT activity in the lung (29). On the other hand, the deficiency of α 1AT is due to oxidation of the amino group of methionine sulfoxide by free radicals present in cigarette smoke (27), or by oxygen radicals which are released by macrophages and polymorphonuclear leukocytes in the lungs (26, 27, 33). Exposure to tobacco smoke causes a considerable increase in the population of pulmonary alveolar macrophages in the lung (7, 26, 27). These macrophages release a chemotactic factor that attracts more macrophages to the lung (26, 27, 34). Moreover, the deficiency of α 1AT partly accounts for the pulmonary emphysema observed in smokers (27). On the other hand, the smoking-induced increase in macrophages and in polymorphonuclear leukocytes leads to a large increase in the release of elastase to the lungs (7, 26, 34, 35). A decrease in elastase activity has been observed as well (36). According to the «two

hit concept», smoking leads to emphysema by both an increase in elastase activity and by a decreased activity of the α 1-antitrypsin antiprotease (25, 34). Cigarette smoking leads to the common centrilobular type of emphysema. Elastases are predominantly released around terminal bronchioles, which represent the site of macrophage accumulation in the lungs of cigarette smokers (26, 37). In contrast, patients with α 1-antitrypsin deficiency are characterized by a basal panlobular type of emphysema. In this condition, lung damage is caused by elastase released from neutrophils which originate from the circulating blood. Neutrophils are likely to be sequestered at the bases because of the greater blood flow to that part of the lungs (26, 38). From these data, it is clear that cigarette smokers with α 1-antitrypsin deficiency are at a very high risk to develop lung disease. It has been shown that median age at onset of dyspnea was only 40 years in α 1AT-deficient patients who smoked vs 53 years in non-smoking deficient cases. Furthermore, life expectancy was significantly shorter in smoking than in non-smoking α 1AT-deficient patients (39).

α 1-ANTITRYPSIN GENE AND ITS ALLELES

α 1-antitrypsin is coded for by a 12 kb long gene which is located on the human chromosome 14 (2, 3, 5, 40, 41). Since two parental genes are codominantly expressed, the α 1AT phenotype, referred to as the Pi (Protease inhibitor) phenotype, is determined by the independent expression of two parental alleles (2, 3, 42-45). The Pi phenotype is determinant of the serum level of α 1AT and of the risk for development of liver or lung disease (Table 2) (2, 3, 7, 38, 43-45). Identification of the Pi phenotype is mainly based on the differences in charge between the different α 1AT molecules. The most useful electrophoresis systems used to distinguish the Pi variants in serum are acid starch gel electrophoresis and isoelectric focusing in polyacrylamide gel at pH 4 to 5 (38, 42-44, 46). A typical pattern on an isoelectric focusing gel for an individual who is homozygous for a normal allele demonstrates five bands, two «major bands» and three «minor bands» (Fig. 3). This microheterogeneity results from post-translational modifications with differences in the carbohydrate side chains and in the length of

TABLE 2. CLASSIFICATION OF α 1-ANTITRYPSIN ALLELIC VARIANTS

Category	Variant name	Serum level mg/dl	Function	Allelic frequency ^{a,b}	Risk for disease	
					Lung	Liver
Normal	M ₁ (Val ²¹³)	150-350	normal	0.44-0.49	No	No
	M ₁ (Ala ²¹³)	150-350	normal	0.20-0.23	No	No
	M ₂	150-350	normal	0.14-0.19	No	No
	M ₃	150-350	normal	0.10-0.11	No	No
	M ₄	150-350	normal	0.01-0.05	No	No
	Others	150-350	normal	rare	No	No
Deficient	Z	15-50	Reduced	0.01-0.02	Yes	Yes
	S	100-200	Normal	0.02-0.04	No	No
	M ^{Heerlen}	<10	?	rare	Yes	No
	M ^{Procidia}	<10	Normal	rare	Yes	No
	M ^{Malton}	<10	?	rare	Yes	Yes
	M ^{Duarte}	<10	?	rare	Yes	Yes
	Others	<10	?	rare	Yes	No
Null	Null ^{granite falls}	0	-	rare	Yes	No
	Null ^{bellingham}	0	-	rare	Yes	No
	Null ^{matawa}	0	-	rare	Yes	No
	Others	0	-	rare	Yes	No
Dysfunctional	Pittsburgh	150-350	altered	rare	No	No

^aFor Caucasians; ^b"Rare" variants, allelic frequencies are less than 0.001 *Acta Clinica Belgica* 48.3 (1993)

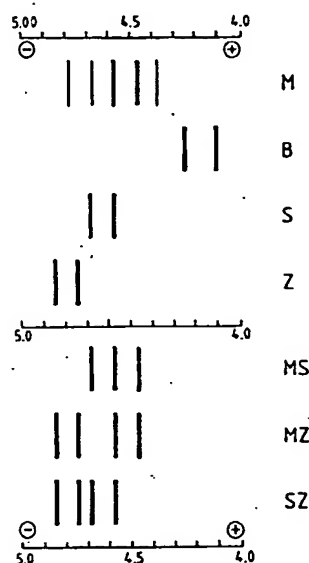


Fig. 3: Schematic illustration of isoelectric focusing of serum at pH 4 to 5.

Upper part: shows the bands corresponding to some α 1AT variants in homozygotes for these variant alleles. Although a homozygote has two identical α 1AT genes, five bands are seen: two major bands (solid bars) and three minor bands (narrow bars). This situation is illustrated for the M-homozygote. For all other conditions, only the major bands are shown. The position of the major bands between cathode and anode determines the latter designated to the variant.

Lower part: shows the major bands in MS, MZ, and SZ heterozygotes. Each allele is responsible for the presence of two major bands. In the MS-heterozygote, one M- and one S-band are in the same position.

the polypeptide chain (1, 2). The position of migration of the major bands between the anode (pH 4) and the cathode (pH 5) determines the letter designated to the α 1AT variant (Fig. 3). The common normal variants migrate in the middle and are referred to as the «M-family» proteins and alleles. Variants that migrate close to the anode are assigned the letters at the beginning of the alphabet. The deficiency variants S and Z on the other hand have a slower mobility and are more cathodal (Fig. 3) (2, 42-44, 46).

Acta Clinica Belgica 48.3 (1993)

Some rare alleles are labeled by a capital letter together with the birth site of the allele (Table 2) (2, 3, 46). With isoelectric focusing, not all allelic variants can be identified. Molecular biology techniques with restriction fragment length polymorphism analysis (2, 47-49) and direct DNA sequence analysis (2, 50, 51) of the α 1AT gene, have allowed the identification of most of the normal and deficient mutations of the α 1AT gene at the DNA level.

With both the isoelectric focusing and molecular biology techniques, more than 75 allelic variants have been reported (2). These α 1AT variants are categorized into four groups: normal, deficient, null, and dysfunctional alleles (Table 2) (2, 3). Normal alleles produce α 1AT molecules with a normal inhibitory function and, if inherited in a homozygous fashion or with another normal allele, with normal α 1AT serum levels. Four allelic variants, M₁ (Ala²¹³), M₁ (Val²¹³), M₂, and M₃ represent more than 95 percent of the known α 1AT variants associated with normal serum levels. Among Caucasians, M₁ (Ala²¹³) is the most common with an allelic frequency of 0.44 to 0.49. In addition to these four normal variants, at least 42 other normal but rarely occurring α 1AT variants have been identified (2, 3). Metabolic studies utilizing radiolabeled α 1AT have estimated that Pi MM individuals produce 34 mg α 1AT/kg body weight per day (52). The half-life of the M protein in plasma is approximately 5 days (52-54). Deficient alleles are associated with lower than normal α 1AT serum levels, whereas the function of the α 1AT molecule may be normal or reduced. The most common deficiency variants are the Z and S mutant (2-4, 7, 43-45). The Z protein differs from the M protein in a single amino acid substitution Glu³⁴² → Lys³⁴². This molecular abnormality leads to a change in the conformation or folding of the nascent α 1AT polypeptide after its translocation into the endoplasmic reticulum. As a result, 85% of the normally synthesized polypeptide is blocked in the endoplasmic reticulum, at a stage prior to final

processing of its carbohydrate (55-58). This retention of Z in the endoplasmic reticulum is followed by polymerization of adjacent Z molecules, resulting in a persistent binding of the nascent polypeptide chain-binding protein. There is a slight increase in the radiolabeled Pi Z α 1AT covalently bound proteins when infused into (54). This difference, however, is not significant for the decreased α 1AT serum levels in individuals (54, 60). Histological cellular accumulation of the protein responds to globular inclusion bodies, predominantly localized in periglobular regions. These globules are strongly PAS (Periodic acid-Schiff) positive, which reflects a high mannose character with a high mannose content. The intracellular α 1AT material is demonstrated by immunohistochemistry with mono-specific antisera (61-63) by electron microscopy (61-63). The newly synthesized Z molecules are secreted into the plasma as the product of the Z-allele gene, representing 15% of the M allele (Tables 2 and 3). In Pi Z individuals, it enhances the biosynthesis of and abnormal (Pi Z) α 1-antitrypsin plasma levels of α 1-antitrypsin are often seen in Pi MZ heterozygotes (62, 65) or under other conditions (62). Not only is the Z protein reduced in the serum, but also the decreased activity as an inhibitor of elastase (66). The single amino acid substitution Glu²⁶⁴ → Val²⁶⁴ that characterizes the S variant does not lead to intracellular retention but to an early intracellular degradation of nascent S polypeptides (2, 3). The S allele gives serum levels equivalent to 60% of the normal (2 and 3). Pi SS individuals have serum levels of 13 to 19 μ M, which

es are labeled by a capital letter the birth site of the allele (Table 2) hisoelectric focusing, not all allelic be identified. Molecular biology with restriction fragment length n analysis (2, 47-49) and direct ce analysis (2, 50, 51) of the α 1AT lowed the identification of most of d deficient mutations of the α 1AT NA level.

the isoelectric focusing and logy techniques, more than 75 allelic : been reported (2). These α 1AT : categorized into four groups: nor- it, null, and dysfunctional alleles (3). Normal alleles produce α 1AT th a normal inhibitory function and, n a homozygous fashion or with al allele, with normal α 1AT serum allelic variants, M_1 (Ala²⁰³), M_1 and M_2 represent more than 95 e known α 1AT variants associated serum levels. Among Caucasians, s the most common with an allelic 0.44 to 0.49. In addition to these variants, at least 42 other normal but ring α 1AT variants have been , 3). Metabolic studies utilizing α 1AT have estimated that Pi MM roduce 34 mg α 1AT/kg body weight . The half-life of the M protein in roximately 5 days (52-54). Deficient ssociated with lower than normal levels, whereas the function of the ule may be normal or reduced. The n deficiency variants are the Z and 4, 7, 43-45). The Z protein differs rotein in a single aminoacid substi- ³⁴² \rightarrow Lys³⁴². This molecular leads to a change in the conforma- g of the nascent α 1AT polypeptide nslocation into the endoplasmic is a result, 85% of the normally polypeptide is blocked in the endo- culum, at a stage prior to final

processing of its carbohydrate side chains (4, 7, 55-58). This retention of Z polypeptides in the endoplasmic reticulum is possibly related to polymerization of adjacent Z molecules (59) or to a persistent binding of the nascent proteins to the polypeptide chain-binding proteins until they are degraded in the endoplasmic reticulum (5, 6). There is a slight increase in the clearance rate of radiolabeled Pi Z α 1AT compared with Pi M proteins when infused into Pi MM individuals (54). This difference, however, does not account for the decreased α 1AT serum levels in deficient individuals (54, 60). Histologically, the intracellular accumulation of the Z polypeptides corresponds to globular inclusions which are predominantly localized in periportal hepatocytes. These globules are strongly PAS (periodic acid-Schiff) positive, which reflects the immature character with a high mannose content (61, 62). The intracellular α 1AT material can also be demonstrated by immunohistochemical staining with mono-specific antisera against α 1AT, and by electron microscopy (61-64). Only 15% of the newly synthesized Z molecules dissociate from PCBP and are secreted into the plasma. As such, the product of the Z-allele gives serum concentrations equivalent to 15% of that of the normal M allele (Tables 2 and 3). Inflammatory activity enhances the biosynthesis of both normal (Pi M) and abnormal (Pi Z) α 1-antitrypsin (13). Normal plasma levels of α 1-antitrypsin are therefore often seen in Pi MZ heterozygotes with liver disease (62, 65) or under other stimulatory conditions (62). Not only is the Z protein present in reduced amounts in the serum, it also has a decreased activity as an inhibitor of the neutrophil elastase (66). The single aminoacid substitution Glu²⁶⁴ \rightarrow Val²⁶⁴ that characterizes the S variant does not lead to intracellular accumulation but to an early intracellular proteolysis of the nascent S polypeptides (2, 3, 7, 67). The product of the S allele gives serum concentrations equivalent to 60% of the normal allele (7) (Tables 2 and 3). Pi SS individuals have serum α 1AT levels of 13 to 19 μ M, which are sufficient to

TABLE 3. RELATIVE SERUM CONCENTRATIONS IN DIFFERENT Pi PHENOTYPES. THE α 1AT SERUM LEVEL IS DETERMINED BY THE INDEPENDENT EXPRESSION OF THE TWO Pi ALLELES. THE PRODUCTS OF THE Z AND S ALLELES GIVE CONCENTRATIONS EQUIVALENT TO 15 AND 60% OF THE NORMAL M ALLELE, RESPECTIVELY.

Phenotype	Mean percentage contribution %
MM	100
MS	80
MZ	57
SS	60
M Null	50
SZ	37
ZZ	15
Z Null	8
Null-Null	0

protect the lung from destruction by elastase. Some other rare deficient variants are given in Table 2. α 1-antitrypsin globules may be seen in the liver parenchymal cells in homo- or heterozygotes for such rare phenotypes as M_{duarte} (68), M_{malton} (69) or for the recently described $M_{cagliari}$ (70).

"Null" α 1AT alleles are rare variants in which no α 1AT is detectable in serum attributable to that gene (2, 3, 44, 49-51, 71, 72). With no α 1AT to protect the lungs, null homozygotes are at a very high risk to develop emphysema (Table 2). The Pittsburgh variant is the only known example of a *dysfunctional* α 1AT variant (Table 2) and is characterized by a severe bleeding diathesis (8).

RISK FOR LUNG AND LIVER DISEASE

Liver injury occurs in α 1AT phenotypes associated with intracellular accumulation of α 1-antitrypsin, as it is the case in Pi Z, Pi M_{malton} , and Pi M_{duarte} phenotypes. In contrast, no liver disease is seen in deficiency phenotypes due to intracellular protein degradation (Pi S) or in Pi Null phenotypes (Table 2). The pathophysiology

Acta Clinica Belgica 48.3 (1993)

of the liver disease is generally considered to be related to the accumulation of α 1AT in the liver parenchymal cells (2-7). This view is supported by experiments in transgenic mice carrying the mutant Z allele of the human α 1AT gene (73, 74). Although these mice secrete normal amounts of endogenous protease inhibitors to the serum, they develop acute liver necrosis and inflammation which is related to the amount of Pi Z α 1AT accumulation in the liver. The emphysema of α 1-antitrypsin deficiency, on the other hand, results from a serum level below 80 mg/dl, which is accompanied by an insufficient protective screen in the lower respiratory tract against the damaging properties of the neutrophil elastase (2, 3, 7, 24, 43-45). As such, the highest risk to develop emphysema is observed in Pi ZZ and in Pi Null-Null phenotypes.

α 1-ANTITRYPSIN DEFICIENCY AND LUNG DISEASE

As outlined in the previous paragraph, all patients with α 1AT serum levels below 80 mg/dl are at risk to develop emphysema. More than 95% of the cases with α 1AT deficiency-related emphysema are Pi ZZ homozygotes. According to population studies performed in different Northern European populations, the prevalence of type Z homozygotes is in the range of 1/1500 to 1/3500 (75-81). The percentage of Pi ZZ patients who develop emphysema is not well known. Although a figure of 80-90% has been quoted (44), it remains entirely possible that emphysema will not develop in a large population of Pi ZZ cases (82). This thesis is supported by the disproportion observed between the number of patients examined and the total number of cases expected in the populations under study (39, 83).

A typical patient with α 1-antitrypsin deficiency develops dyspnoea on exertion between ages 30 to 40 (2, 3, 44, 45, 83, 84). The disease is markedly accelerated by smoking. In the study of Larsson (39), median age at onset of dyspnea in *Acta Clinica Belgica* 48.3 (1993)

Pi Z smokers was 40 yrs, as compared to 53 yrs in non-smokers. Although it is infrequent as an initial symptom, approximately 50% of Pi ZZ patients develop a cough and recurrent pulmonary infections as signs of chronic bronchitis during their further evolution (44). The chest film shows flattened diaphragms and hyperinflated lungs with reduced peripheral vasculature, particularly in the lower lobes (Fig. 4) (44, 45, 82, 84). Pulmonary function tests are consistent with severe emphysema. Severe expiratory airflow limitation is a common feature and is attributable to airway collapse from loss of pulmonary elastic recoil (44, 45, 82). In pathologic studies, the lungs are characterized by a panlobular type of emphysema (44, 45, 75). This picture of emphysema in α 1AT deficient patients is in marked contrast with the commonly acquired form of emphysema in smoking Pi MM patients. The latter group is characterized by initial signs of chronic bronchitis, by an upper lung zone distribution, and by centrilobular type of emphysema.



Fig. 4: Chest X-ray of a Pi ZZ homozygote with lung emphysema. Notice the flattened diaphragms and the hyperinflated lungs, mainly in the lower lobes. This patient died of respiratory insufficiency at 58 yrs of age. At autopsy, a panlobular type of emphysema was found.

The question arises whether patients, with lesser degrees of increased risk to develop emphysema. Theoretically, *SZ* heterozygotes of about one third of the normal level, and, therefore, appear to be elastic recoil, hypoventilation have, indeed been asymptomatic *SZ* cases (85) experience of Hutchison et al the *SZ* phenotype per se is not a risk. Only 1 of their 11 non-*SZ* developed emphysema. One patient had a history of cigarette smoking at onset of symptoms observed in emphysematous phenotype (82, 86). As for *zygotes*, there is no evidence that predisposes to the development of lung disease in *MZ* heterozygotes. Subject of exhaustive investigation of studies have been performed on prevalence of Pi MZ phenotype patients with chronic obstructive pulmonary disease compared to control patients. In these studies, it can be concluded that prevalence of Pi MZ is higher than in controls. Secondly, Pi MZ patients selected from a community study and their pulmonary function compared with that of Pi MM cases from the same population (45, 87, 90-93). According to these studies, no difference is found in the primary symptoms or spirometric values among Pi MZ and Pi MM cases. Studies of pulmonary function have been performed in groups of Pi MZ cases. Characteristic expiratory flow rate have been reported (94) as well as in adult heterozygotes. A recent study, however, showed no difference in lung elasticity between Pi MZ and Pi MM individuals (96). In a study by al. (97), lung function of non-smoking subjects did not differ from that of Pi M controls.

of the liver disease is generally considered to be related to the accumulation of α 1AT in the liver parenchymal cells (2-7). This view is supported by experiments in transgenic mice carrying the mutant Z allele of the human α 1AT gene (73, 74). Although these mice secrete normal amounts of endogenous protease inhibitors to the serum, they develop acute liver necrosis and inflammation which is related to the amount of Pi Z α 1AT accumulation in the liver. The emphysema of α 1-antitrypsin deficiency, on the other hand, results from a serum level below 80 mg/dl, which is accompanied by an insufficient protective screen in the lower respiratory tract against the damaging properties of the neutrophil elastase (2, 3, 7, 24, 43-45). As such, the highest risk to develop emphysema is observed in Pi ZZ and in Pi Null-Null phenotypes.

α 1-ANTITRYPSIN DEFICIENCY AND LUNG DISEASE

As outlined in the previous paragraph, all patients with α 1AT serum levels below 80 mg/dl are at risk to develop emphysema. More than 95% of the cases with α 1AT deficiency-related emphysema are Pi ZZ homozygotes. According to population studies performed in different Northern European populations, the prevalence of type Z homozygotes is in the range of 1/1500 to 1/3500 (75-81). The percentage of Pi ZZ patients who develop emphysema is not well known. Although a figure of 80-90% has been quoted (44), it remains entirely possible that emphysema will not develop in a large population of Pi ZZ cases (82). This thesis is supported by the disproportion observed between the number of patients examined and the total number of cases expected in the populations under study (39, 83).

A typical patient with α 1-antitrypsin deficiency develops dyspnoea on exertion between ages 30 to 40 (2, 3, 44, 45, 83, 84). The disease is markedly accelerated by smoking. In the study of Larsson (39), median age at onset of dyspnea in *Acta Clinica Belgica* 48.3 (1993)

Pi Z smokers was 40 yrs, as compared to 53 yrs in non-smokers. Although it is infrequent as an initial symptom, approximately 50% of Pi ZZ patients develop a cough and recurrent pulmonary infections as signs of chronic bronchitis during their further evolution (44). The chest film shows flattened diaphragms and hyperinflated lungs with reduced peripheral vasculature, particularly in the lower lobes (Fig. 4) (44, 45, 82, 84). Pulmonary function tests are consistent with severe emphysema. Severe expiratory airflow limitation is a common feature and is attributable to airway collapse from loss of pulmonary elastic recoil (44, 45, 82). In pathologic studies, the lungs are characterized by a panlobular type of emphysema (44, 45, 75). This picture of emphysema in α 1AT deficient patients is in marked contrast with the commonly acquired form of emphysema in smoking Pi MM patients. The latter group is characterized by initial signs of chronic bronchitis, by an upper lung zone distribution, and by centrilobular type of emphysema.

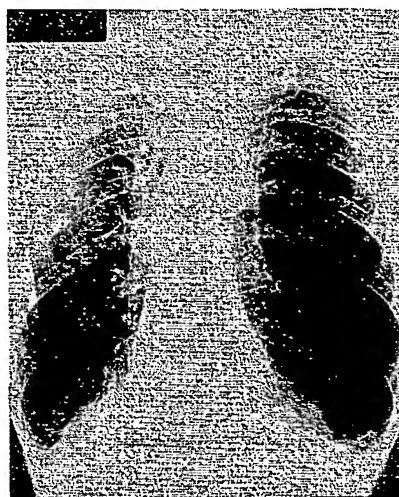


Fig. 4: Chest X-ray of a Pi ZZ homozygote with lung emphysema. Notice the flattened diaphragms and the hyperinflated lungs, mainly in the lower lobes. This patient died of respiratory insufficiency at 58 yrs of age. At autopsy, a panlobular type of emphysema was found.

The question arises whether patients, with lesser degrees of increased risk to develop emphysema. Theoretically, *SZ heterozygotes* of about one third of the normal and, therefore, appear to be elastic recoil, hypoventilation have indeed been asymptomatic SZ cases (85) experience of Hutchison et al the SZ phenotype per se may risk. Only 1 of their 11 non-SZ developed emphysema. A patient had a history of cigarette age at onset of symptoms observed in emphysematous phenotype (82, 86). As for *zygotes*, there is no evidence predisposes to the development (7, 45, 82). The possible prelung disease in *MZ heterozygotes* subject of exhaustive investigation of studies have been performed prevalence of Pi MZ phenotype patients with chronic obstructive compared to control patients these studies, it can be concluded prevalence of Pi MZ is higher than controls. Secondly, Pi MZ patients selected from a community study and their pulmonary function with that of Pi MM cases from the same population (45, 87, 90-93). According to no difference is found in the primary symptoms or spirometry among Pi MZ and Pi MM cases of pulmonary function have been compared groups of Pi MZ cases. Characteristic expiratory flow rate have been compared (94) as well as in adult heterozygotes. A recent study, however, showed no difference in lung elasticity between Pi MM individuals (96). In a study by al. (97), lung function of non-smoking subjects did not differ from the Pi M controls.

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X-ray of a Pi ZZ homozygote with lung Notice the flattened diaphragms and the lungs, mainly in the lower lobes. This of respiratory insufficiency at 58 yrs of y, a panlobular type of emphysema was

The question arises whether heterozygous patients, with lesser degrees of deficiency, are at increased risk to develop lung damage. Theoretically, SZ heterozygotes have serum levels of about one third of the normal value (Table 3) and, therefore, appear to be at risk. A loss of elastic recoil, hypoventilation and hypoperfusion have indeed been demonstrated in asymptomatic SZ cases (85). However, from the experience of Hutchison et al. (86), it appears that the SZ phenotype per se may carry little or no risk. Only 1 of their 11 non-index cases with Pi SZ developed emphysema. This particular patient had a history of cigarette smoking and his age at onset of symptoms was similar to that observed in emphysematous patients of normal phenotype (82, 86). As for the Pi MS heterozygotes, there is no evidence that this phenotype predisposes to the development of lung disease (7, 45, 82). The possible predisposition toward lung disease in MZ heterozygotes has been the subject of exhaustive investigation. Three types of studies have been performed. Firstly, the prevalence of Pi MZ phenotype was studied in patients with chronic obstructive lung disease as compared to control patients (45, 87-89). From these studies, it can be concluded that the prevalence of Pi MZ is higher in patients than in controls. Secondly, Pi MZ heterozygotes were selected from a community or working population and their pulmonary function was compared with that of Pi MM cases from the same population (45, 87, 90-93). According to these studies, no difference is found in the prevalence of pulmonary symptoms or spirometric abnormalities among Pi MZ and Pi MM cases. Thirdly, studies of pulmonary function have been carried out in groups of Pi MZ cases. Changes in the forced expiratory flow rate have been observed in children (94) as well as in adult heterozygotes (95). A recent study, however, showed no significant difference in lung elasticity between Pi MZ and Pi MM individuals (96). In a study of Larsson et al. (97), lung function of non-smoking Pi MZ subjects did not differ from that of non-smoking Pi M controls.

However, a mild impairment in pulmonary function was found in MZ subjects who had smoked. It can be concluded from these different studies that the MZ phenotype per se carries little or no risk to develop lung emphysema. Overall, the risk in heterozygous SZ and MZ patients seems highly influenced by environmental factors such as cigarette smoking and occupational air pollution. These factors are to be avoided, not only in homozygote deficient cases, but also in less deficient heterozygotes.

NEONATAL LIVER DISEASE IN α 1-ANTITRYPSIN DEFICIENCY

An association between Pi ZZ phenotype α 1AT deficiency, neonatal cholestasis, and childhood cirrhosis has been established in several series (76, 98-106). Sveger (76, 103, 104) studied 127 Pi ZZ patients among 200,000 newborns screened in Sweden. Fourteen out of these 127 children (11%) had neonatal cholestasis and 8/127 (6%) showed clinical evidence of liver disease without jaundice. Seventy-three percent of the children who had no clinical symptoms of liver disease had abnormal serum alanine aminotransferase levels at 6 months of age. At 12-yr follow-up, three children had died with liver cirrhosis, corresponding to a risk of death from cirrhosis during childhood of 2-3%. At that age, alanine aminotransferase was abnormal in 33% of the Pi ZZ children who had suffered neonatal liver disease, and only in 14% of the previously healthy Pi ZZ children. This favorable outcome is in contrast with the much more dismal prognosis described by others. In the series of Ghisham and Greene (106) out of 15 Pi ZZ children with neonatal cholestasis, three underwent liver transplantation, two died from complicated liver cirrhosis, and three had histological evidence of cirrhosis. Of the remaining 7 cases (47%), all continued to have an enlarged liver and spleen, and abnormal liver tests. In the follow-up study of Psa-charopoulos et al. (102) on 67 Pi ZZ children who presented with neonatal hepatitis,

Acta Clinica Belgica 48.3 (1993)

19 (28%) died of liver cirrhosis, 19 (28%) had established cirrhosis, and 14 (21%) had persistent clinical and biochemical evidence of liver disease. Factors which may carry a poor prognosis in children with α 1AT deficiency consist of the histological documentation of marked periportal fibrosis with ductular proliferation (107, 108), the persistent elevation of liver enzymes beyond 1 yr of age (100), and the presence of severe liver disease in an affected sibling (76, 102). In the series of Ghisham (106), however, the evolution of the liver disease could not be correlated with the early histological picture. Since only a minority of Pi ZZ children present with neonatal cholestasis, additional factors have been looked for which might be important for the pathogenesis of the liver disease. In view of the male predominance and of the tendency of the liver disease to occur in certain families (76, 102, 106, 109), additional genetic and hormonal factors have been proposed. Moreover, a protective effect of breast milk feeding has been suggested (109, 110).

Clinically, neonatal cholestasis begins at birth or in the first weeks of life. Symptoms and signs consist of jaundice, dark urine, pale stools, hepatomegaly, splenomegaly, failure to gain weight, and hemorrhagic complications due to deficiency of Vitamin K. Jaundice usually recovers within 6 months (100, 102, 107). The findings of intrauterine growth retardation (76, 111), of neonatal cirrhosis (111), and of bile duct destruction in a 20-wk old Pi ZZ fetus (112) all suggest that the damaging effect of α 1AT deficiency to the liver may already occur during intrauterine life. According to Hadchouel and Gautier (107), three morphological patterns of hepatic alterations can be found in children with Pi ZZ-related neonatal cholestasis. A first group mainly shows neonatal hepatitis with hepatocellular damage and cholestasis, with a variable degree of giant cell transformation, and relatively little infiltration with inflammatory cells. A second group mainly presents with marked periportal fibrosis and bile duct proliferation. Hepatic ductular hypoplasia is the most remarkable finding in a third

Acta Clinica Belgica 48.3 (1993)

group. Paucity of the interlobular bile ducts as well as extrahepatic bile duct hypoplasia have also been observed by others (112-115).

Neonatal liver disease has mostly been observed in Pi ZZ homozygotes. Very rarely, however, neonatal hepatitis or bile duct hypoplasia have been reported in SZ (111, 113, 116-119) or in MZ (105) heterozygotes. In these cases, neonatal cholestasis had a much better prognosis than in Pi ZZ homozygotes (105, 119).

Prenatal identification of the Pi phenotype can be performed on chromosomal DNA purified from amniotic or chorionic villus cells, by using 32 P-labeled synthetic oligonucleotides (120-123).

ADULT LIVER DISEASE IN α 1-ANTITRYPSIN DEFICIENCY

Adult patients with homozygous Pi Z α 1AT deficiency are at increased risk to develop cirrhosis and hepatocellular carcinoma (39, 124-127). Larsson found liver cirrhosis in only 2% of 104 Pi ZZ cases between 20 and 50 years old, but in 19% of 142 homozygotes over the age of 50 (39). In a case-control study based on all autopsied cases of α 1AT deficiency in Malmö over the 20-yr period from 1963 to 1982, a causal association between α 1AT deficiency and cirrhosis (odds ratio 7.8) and primary liver cancer (odds ratio 20) could be demonstrated. These associations, however, were only significant for males (125).

Clinically, liver disease in adult α 1AT deficient patients mainly presents in males over the age of 50 (39, 125-128). A history of neonatal hepatitis is very rare (126, 128). Presenting symptoms consist of consequences of portal hypertension with progressive ascites and variceal bleeding (126). Laboratory findings are characterized by only very modest elevations of serum transaminases and bilirubin (126, 128). Most patients show increased serum levels of alkaline phosphatase and of gamma globulin (126, 128). The prognosis is generally poor with a mean survival of 2 years after diagnosis (126).

Not only Pi ZZ homozygous Pi MZ and SZ patients are at increased risk to develop adult hepatocellular carcinoma (5).

TREATMENT OF α 1-ANTITRYPSIN DEFICIENCY

The most important preventive measure for patients with α 1AT deficiency is to avoid smoking. Cigarette smoking increases the destructive lung disease and shortens the longevity of the patient. The effect of smoking against the disease seems also to be more pronounced in deficient Pi MZ and SZ heterozygotes than in Pi ZZ homozygotes. Pulmonary infections as well as reversible bronchospastic conditions are more amenable to treatment. Theoretically, androgen danazol and the anti-estrogen tamoxifen can be used to augment α 1AT levels (136-139). However, in homozygous patients this is too slow to be clinically significant. Moreover, danazol acts by stimulating α 1AT synthesis, but they may increase the intracellular levels of the mutant Z α 1AT, which may even increase the risk to develop liver disease. Human plasma α 1AT is available for treatment of α 1AT deficient patients (Weekly 9, 10, 23, 140-142). Intravenous infusions as well

Fig. 5

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y of the interlobular bile ducts as hepatic bile duct hypoplasia have served by others (112-115).

liver disease has mostly been Pi ZZ homozygotes. Very rarely, neonatal hepatitis or bile duct hypoplasia reported in SZ (111, 113, 116-119) or heterozygotes. In these cases, cirrhosis had a much better prognosis than homozygotes (105, 119).

Identification of the Pi phenotype can be done on chromosomal DNA purified from chorionic villus cells, by using synthetic oligonucleotides (120-123).

LIVER DISEASE IN α 1-ANTI-TRYPsin DEFICIENCY

Patients with homozygous Pi Z α 1AT are at increased risk to develop cirrhosis and cellular carcinoma (39,124-127). End liver cirrhosis in only 2% of 104 between 20 and 50 years old, but in homozygotes over the age of 50 (39). A control study based on all autopsied Pi Z deficiency in Malmö over the 20 years from 1963 to 1982, a causal association between α 1AT deficiency and cirrhosis (odds ratio 20) was demonstrated. These associations were only significant for males (125).

Liver disease in adult α 1AT deficient patients presents in males over the age of 28). A history of neonatal hepatitis (126, 128). Presenting symptoms and consequences of portal hypertension like ascites and variceal bleeding. Laboratory findings are characterized by modest elevations of serum transaminases, bilirubin (126, 128). Most patients had serum levels of alkaline phosphatase and gamma globulin (126,128). The prognosis is generally poor with a mean survival after diagnosis (126).

Not only Pi ZZ homozygotes but also heterozygous Pi MZ and SZ patients are at increased risk to develop adulthood cirrhosis and hepatocellular carcinoma (59, 129-135).

TREATMENT OF α 1-ANTITRYPSIN DEFICIENCY

The most important preventive measure in patients with α 1AT deficiency is avoidance of smoking. Cigarette smoking markedly accelerates the destructive lung disease and significantly shortens the longevity of these patients. Advice against smoking seems also indicated in less deficient Pi MZ and SZ heterozygotes. Bronchopulmonary infections as well as a potentially reversible bronchospastic component are amenable to treatment. Theoretically, the weak androgen danazol and the estrogen antagonist tamoxifen can be used to augment α 1AT serum levels (136-139). However, the response in homozygous patients is too small and variable to be clinically significant. Moreover, these drugs act by stimulating α 1AT synthesis. Therefore, they may increase the intracellular accumulation of the mutant Z α 1AT, which might lead to an even greater risk to develop liver disease. Purified human plasma α 1AT is available for the treatment of α 1AT deficient patients with emphysema. Weekly (9, 10, 23, 140-142) or monthly (143) intravenous infusions as well as aerosol therapy

with α 1-antitrypsin (144) improves the concentration of α 1AT and the neutrophil elastase inhibitory capacity in broncho-alveolar lavage fluid. There are, however, several potential complications of this form of therapy. The elastase- α 1AT complexes which are formed during this type of therapy are chemotactic (145, 146) and may attract neutrophils into various tissues, including the lungs. Moreover, these complexes act as a mediator of feedback induction of α 1AT biosynthesis after their interaction with the «serpin-enzyme complex» receptors (5, 19, 20). This effect again may lead to an increased intracellular accumulation of variant α 1AT in the liver parenchymal cells, and thus to an increased propensity toward liver cell injury. Recombinant α 1-antitrypsin that may contain amino acid modifications to produce α 1AT more resistant to oxidation in the reactive center of the molecule, should be available in the future (4,147). Newly discovered low-molecular-weight cephalosporin neutrophil elastase inhibitors have been shown to be potent, highly specific irreversible inhibitors of human neutrophil elastase in vitro (148,149) as well as in an in vivo lung haemorrhage model in the hamster (150). In the future, synthetic elastase inhibitors may prove to be of therapeutic value in α 1AT deficient patients.

End-stage liver disease in α 1AT deficient children and adults can be treated by orthotopic liver transplantation (151, 152). In the series of

α 1-ANTITRYPSIN AND ITS DEFICIENCY

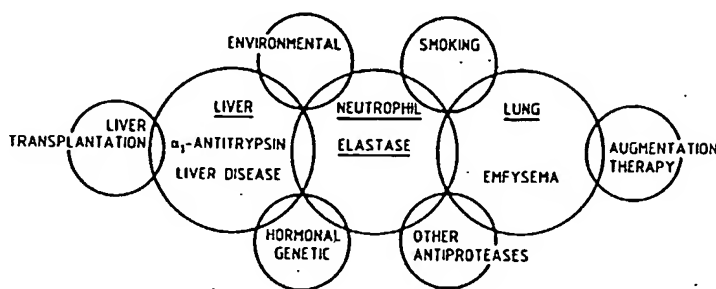


Fig. 5

Acta Clinica Belgica 48.3 (1993)

Esquivel et al. (152), a 5-yr actuarial survival of 83% and 60% is reported in pediatric and adult recipients, respectively. After liver transplantation, the phenotype of α 1AT in the serum of the recipient changes to that of the donor (151, 153).

A porto-systemic shunt operation may be considered in well-selected α 1AT deficient children with only mild parenchymal liver disease but with severe portal hypertension (5, 154).

CONCLUSION

α 1-antitrypsin is mainly produced by liver parenchymal cells and is the major inhibitor of the neutrophil elastase. In normal conditions, it protects the lungs against proteolytic damage by this enzyme. The pulmonary disease of α 1AT deficiency is a direct consequence of a deficient α 1AT production and can be treated by replacement therapy. Additional factors such as cigarette smoking and the presence or absence of other antiproteases in the lung may modify the clinical picture of the lung disease in these patients. Approximately 20% of α 1AT deficient individuals develop a spectrum of liver diseases which are characterized by the accumulation of α 1AT inclusions in the parenchymal cells of the liver. Engorgement of the liver cells with α 1AT is the most likely cause of the liver disease. Other factors, such as hormonal, genetic and environmental influences may modify the outcome of the disease. The only current therapy for advanced liver disease in these patients is liver transplantation.

ACKNOWLEDGEMENTS

The author wishes to thank Miss Agnes Goethuys who typed the manuscript.

SAMENVATTING

1. α 1-antitrypsine is een inhibitor van het neutrofiele elastase enzyme. Het maakt deel uit van een groep van *Acta Clinica Belgica* 48.3 (1993)

structureel verwante eiwitten die gemeenschappelijk met de term «serpins» worden aangeduid, hetgeen staat voor «serine protease inhibitors». Het Methionine³⁵⁸ van α 1-antitrypsine is bepalend voor de specificiteit van binding aan elastase.

2. Het normale M-type α 1-antitrypsine wordt vooral aangemaakt in de parenchymcellen van de lever en wordt uitgescheiden in het bloed. Abnormaal Z- α 1-antitrypsine wordt weerhouden in het endoplasmatisch reticulum van de levercellen. Dit leidt tot een opstapeling van het abnormale proteïne in de levercellen en tot een gedaalde concentratie in het bloed.

3. In normale omstandigheden beschermt het α 1-antitrypsine de longen tegen de proteolytische activiteit van het elastase. Het onevenwicht tussen protease- en antiprotease activiteit is verantwoordelijk voor het optreden van longemfyseem bij ernstige α 1-antitrypsine deficiëntie en bij rokers. Het verklaart ook de snellere achteruitgang van de longfunctie bij α 1-antitrypsine deficiënte patiënten die roken. Roken dient absoluut vermeden te worden bij deze patiënten. Patiënten met α 1-antitrypsine deficiëntie en emfyseem kunnen behandeld worden met α 1-antitrypsine dat afkomstig is van menselijk plasma.

4. De intracellulaire opstapeling van het abnormale Z- α 1-antitrypsine in de levercellen kan leiden tot een leveraandoening. Deze kan bestaan uit neonatale cholestase of uit levercirrose en primaire leverkanker bij volwassenen. Patiënten met leverinsufficiëntie kunnen behandeld worden met levertransplantatie. Deze behandeling leidt tot een verandering van het α 1-antitrypsine fenotype van de patiënt.

5. De diagnose van α 1-antitrypsine kan worden gesteld aan de hand van de serum concentratie van α 1-antitrypsine en van het aantreffen van «periodic acid Schiff» positieve korreltjes in de levercellen. Iso-elektrische focusering van het plasma laat toe het fenotype van α 1-antitrypsine te bepalen. Dit fenotype is bepalend voor de plasmaconcentratie en voor de eventuele ontwikkeling van long- en leverletsels.

RESUME

1. L' α 1-antitrypsine est une antiprotéase qui inhibe l'élastase des neutrophiles et qui appartient à une famille d'inhibiteurs des protéinases structurellement apparentés à la sérine (serpins). Sa spécificité pour l'élastase est déterminée par son résidu méthionine³⁵⁸.

2. L' α 1-antitrypsine normale est synthétisée principalement par les cellules parenchymateuses du foie et est sécrétée dans le plasma. Le mutant Z anormal est retenu dans le réticulum endoplasmique et entraîne une accumulation intracellulaire et une diminution de la concentration plasmatique.

3. Dans des conditions normales, l' α 1-antitrypsine protège les poumons vis-à-vis de l'action de l'élastase des neutrophiles. Un déséquilibre entre l'antiprotéase et la protéase conduit à l'apparition d'un emphyseme dans les cas de déficience en α 1-antitrypsine et chez les fumeurs. Cela explique également la progression rapide de la maladie chez les patients fumeurs déficients en α 1-antitrypsine. Le tabac doit donc être évité chez les patients en α 1-antitrypsine. Un traitement par α 1-antitrypsine humaine est indiqué chez les patients avec une déficience en α 1-antitrypsine porteurs d'un emphyseme.

4. L'accumulation intracellulaire du mutant Z anormal dans les hépatocytes peut entraîner une hépatopathie qui va de la cholestase néonatale à la cirrhose chez l'adulte et au carcinome du foie. Les hépatopathies terminales peuvent être traitées par transplantation hépatique, qui entraîne un changement de phénotype.

5. Le diagnostic de l'affection par déficience en α 1-antitrypsine repose sur la mesure de la concentration sérique basse d' α 1-antitrypsine et sur la présence de globules PAS-positifs dans les cellules du foie. L'électrophorèse du sérum détermine le phénotype de l'antitrypsine. L'isoélectrofocalisation du plasma permet de déterminer le phénotype de l' α 1-antitrypsine. Ce phénotype est déterminant pour la concentration sérique de même que le risque d'une affection pulmonaire ou hépatique.

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le omstandigheden beschermt het α 1- longen tegen de proteolytische activiteit . Het onevenwicht tussen protease- en tiviteit is verantwoordelijk voor het ongemfyseem bij ernstige α 1-antitryp- en bij rokers. Het verklaart ook de uitgang van de longfunctie bij α 1- ficiënte patiënten die roken. Roken vermeden te worden bij deze patiënten. α 1-antitrypsine deficiënte en emfyseem deld worden met α 1-antitrypsine dat in menselijk plasma.

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itrypsine est une antiprotéase qui inhibe neutrophiles et qui appartient à une iteurs des protéinases structurellement a sérine (serpines). Sa spécificité pour éterminée par son résidu méthionine³⁵⁸.

2. L' α 1-antitrypsine normale (de type M) est synthétisée principalement dans les cellules parenchymateuses du foie et est transportée vers le plasma. Le mutant Z anormal de l' α 1-antitrypsine est retenu dans le réticulum endoplasmique, ce qui entraîne une accumulation intra-cellulaire de ces molécules et une diminution importante des taux plasmatiques.

3. Dans des conditions normales, l' α 1-antitrypsine protège les poumons vis-à-vis de l'activité protéolytique de l'élastase des neutrophiles. Un déséquilibre protéase/ antiprotéase dans le poumon va entraîner le développement d'un emphysème dans les déficiences sévères en α 1-antitrypsine et chez les fumeurs, et rend compte de la progression rapide des maladies pulmonaires chez les patients fumeurs déficients en α 1-antitrypsine. Le tabac doit donc être évité chez les patients déficients en α 1-antitrypsine. Un traitement de substitution par un dérivé de l' α 1-antitrypsine plasmatique semble indiqué chez les patients avec déficience en α 1-antitrypsine porteurs d'un emphysème.

4. L'accumulation intracellulaire d' α 1-antitrypsine Z-anormale dans les hépatocytes peut entraîner une hépatopathie qui va de la cholestase néonatale à la cirrhose chez l'adulte et au carcinome hépatocellulaire. Les hépatopathies terminales peuvent être traitées par transplantation hépatique, qui entraîne une conversion phénotypique.

5. Le diagnostic des affections liées à une déficience en α 1-antitrypsine repose sur la présence de concentration sérique basse d' α 1-antitrypsine et de globules PAS-positifs dans les cellules hépatocytaires. L'électrophorèse du sérum détermine le phénotype de cet inhibiteur des protéases. Celui-ci est déterminé par l'expression indépendante de deux allèles parentaux pour l' α 1-antitrypsine. Ce phénotype détermine le taux sérique de même que le risque de développement d'une affection pulmonaire ou hépatique.

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Acta Clinica Belgica 48.3 (1993)

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